

Effects of Δ^9 -Tetrahydrocannabinol on Excitable Membranes and Neuromuscular Transmission

NURAN M. KUMBARACI¹ AND WILLIAM L. NASTUK

Department of Physiology, College of Physicians and Surgeons, Columbia University, New York, New York 10032

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SUMMARY

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The effects of Δ^9 -tetrahydrocannabinol (THC) on excitable membranes and neuromuscular transmission were investigated utilizing the isolated sciatic nerve-sartorius muscle preparation of the frog. Neuromuscular transmission was depressed upon bath application of 30×10^{-6} M THC. Records taken at junctional regions showed that upon nerve stimulation only 50% of the muscle fibers tested elicited a propagated action potential. These action potentials showed a reduction in the overshoot, the maximum rate of rise, and the maximum rate of fall. For the remainder of the muscle fibers, 10% showed no end-plate potentials and 40% gave subliminal end-plate potentials with amplitudes ranging from 5 to 30 mV. At blocked junctions the miniature end-plate potential amplitude was increased and the frequency was reduced. The resting potential of THC-treated fibers did not differ from that of controls. Muscle fiber action potentials recorded at nonjunctional regions showed a decrease in the overshoot, in the maximum rate of rise, and in the maximum rate of fall. The above changes persisted after THC was removed from the bathing solution. THC caused a small but significant increase in postjunctional membrane sensitivity to carbamylcholine. At junctional regions, the membrane input resistance and membrane time constant were unchanged. THC caused a decrease in the quantal content, and if drug application was prolonged, a complete blockade of quantal release was produced. Thus, THC blocks neuromuscular transmission by depressing the release of acetylcholine from presynaptic nerve terminals. In addition, THC depresses the ionic conductance mechanisms which underlie the propagation of action potentials in excitable membranes.

INTRODUCTION

Δ^9 -Tetrahydrocannabinol (THC)² is the major pharmacologically active component of *Cannabis sativa*. Several studies have shown that THC has an action on the peripheral nervous system. THC depresses the twitch response of the transmurally stimulated guinea pig ileum at concentrations of 1.6×10^{-7} to 3×10^{-6} M and recovery is slow after removal of the drug (1, 2). An initial potentiation of the twitch response was observed in rat phrenic

nerve-diaphragm preparations, with blockade to indirect stimulation developing after prolonged application of 10 - $100 \mu\text{M}$ THC (3). Hoekman *et al.* (4) observed that the amplitude and frequency of the miniature end-plate potential (MEPP) were increased and the end-plate potential (EPP) duration was prolonged by THC, with no significant effect on the resting membrane potential (RP) or cholinesterase activity. In desheathed rabbit vagus nerve, Byck and Ritchie (5) found that $30 \mu\text{M}$ THC produced an $8.7 \pm 2.8\%$ fall in the amplitude of the compound action potential and recovery was incomplete many hours after drug removal.

The aforementioned studies show that THC depresses the twitch response of muscle to indirect stimulation and it also depresses action potentials in peripheral nerve. However, the exact sites and mechanisms underlying these effects are unknown. In the present study, single fiber electrophysiological methods were used to investigate the action of THC at the neuromuscular synapse. It was found that THC causes changes in the electrically

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¹ Present address: Department of Chemistry, Stevens Institute of Technology, Hoboken, New Jersey 07030.

² Abbreviations used: ACH, acetylcholine; AP, action potential; CARB, carbamylcholine; EPP, end-plate potential; MEPP, miniature end-plate potential; MRF, maximum rate of fall of AP; MRR, maximum rate of rise of AP; OS, overshoot of AP; PJM, postjunctional membrane; THC, Δ^9 -trans-tetrahydrocannabinol.

excitable conductile membrane of muscle and reduces the AP-evoked release of acetylcholine (ACH) from presynaptic nerve terminals. A preliminary account of these findings has been given (6).

METHODS

Experiments were done *in vitro* using sciatic nerve-sartorius muscle preparations of the frog (*Rana pipiens*). The tissue was bathed at room temperature (18–20°C) in Ringer solution of the following composition: 112.4 mM Na⁺, 2.5 mM K⁺, 1.8 mM Ca²⁺, 117.1 mM Cl⁻, 3.0 mM Hepes buffer at pH = 7.4. THC was obtained as a 3 mM solution in absolute ethanol. A test solution containing THC was prepared immediately before the experiment by adding an appropriate quantity of stock THC to Ringer solution. Most experiments were done using Ringer solution containing 30 μ M THC and 1% ethanol. Control experiments were done on muscles bathing in Ringer solution and in Ringer solution containing 1% ethanol.

Muscle fiber transmembrane potentials were recorded by means of standard intracellular microelectrode techniques. The sciatic nerve was stimulated with supramaximal nerve stimuli (2 $\times$ threshold) in 0.1-ms rectangular pulses and APs were recorded during conduction along the fiber. The THC test solution was bath applied and records were taken for 3 h. The tissue was then washed several times with Ringer solution and the measurements were repeated. After THC application the threshold stimulus strength was elevated, thus applied stimuli were readjusted to be 2 $\times$ the new threshold value.

Postjunctional membrane (PJM) sensitivity was tested by microperfusing the end-plate regions with Ringer solutions containing 20 μ M carbamylcholine (CARB). Perfusion was achieved by a gravity-driven flow of liquid through a glass pipet having a tip diameter of 50–70 μ m. A single muscle fiber was impaled at the junctional region with a recording microelectrode. Thereafter the junction was perfused with a solution of CARB dissolved in Ringer solution of the same composition as that bathing the preparation. The resulting depolarization produced during 30 s of CARB perfusion was recorded. The microperfusion pipet was withdrawn, the preparation was washed with CARB-free bathing solution, and 10 min later the same procedure was repeated at another junctional region.

Membrane input resistance was measured by applying current pulses produced by a constant-current isolator. Two intracellular microelectrodes were positioned 50 μ m apart in a single muscle fiber at the junctional region. One electrode was used for passing 400-ms hyperpolarizing current pulses of constant amplitude. The resulting changes in membrane potential (approx 5–10 mV) were recorded with the second electrode. Resistance was calculated as a quotient of the incremental change in membrane potential and the applied current which produced the change. The time constant was measured as the time to achieve 84% of the maximum membrane potential change (7).

Quantal content was determined by the "method of failures" (8). Transmitter release was lowered by using a modified Ringer solution in which calcium was reduced

to 0.9 mM and 4.0 mM magnesium was added. Preparations were equilibrated in this solution for 1 h. A muscle fiber was then impaled at the junctional region and the failure rate in quantal release was determined by using a low-frequency (1-Hz) train of stimuli. After the control quantal content was determined, the junction was microperfused for 10 min with 30 μ M THC dissolved in the previous equilibration medium, then the microperfusion pipet was withdrawn and the quantal content was again determined. (Records were taken at various elapsed time intervals following the application of THC because the action of the drug develops slowly.) Thereafter this experiment was repeated at more distant junctional regions of the muscle not already exposed to THC.

A statistically significant difference between two means was determined by carrying out a standard non-paired *t*-test analysis.

RESULTS

Preliminary studies using 0.3, 3.0, 30, and 300 μ M THC-containing Ringer solutions showed that significant changes were observed in 30 μ M THC within 3 h. With 0.3 and 3.0 μ M THC, significant effects were observed only after the drug had been applied for 5 h or longer. With 300 μ M THC, the contractile system of muscle fibers became active and reliable electrical recordings could not be taken because of mechanical disturbances. To avoid this artifact and to reduce the time of application of THC, all experiments were conducted in a 30 μ M THC-containing test solution. Recordings were taken at 15-min intervals during a 10- to 180-min period after the start of the application of the drug, and again after removal from the bath and rinsing. Control records were taken at the beginning of the experiments with the preparation bathing in Ringer solution.

Effects of THC on muscle fiber action potentials. Neurally initiated APs were recorded along nonjunctional regions of the fiber (Table 1). Resting membrane potentials of THC-treated fibers did not differ from those of controls. However, single supramaximal nerve stimulation failed to evoke a propagated AP in 39% of the fibers. Those APs which were successfully initiated showed, as they were conducted along the muscle fiber, a 9% decrease in the overshoot (OS), a 17% decrease in the maximum rate of rise (MRR), and a 22% decrease in the maximum rate of fall (MRF). After the removal of THC, 45% of the fibers failed to respond to neural stimulation. In those fibers where an AP was elicited, there was a 9% decrease in OS and an 18% decrease in MRR and MRF.

Effects of THC on neuromuscular junctions. Data from records made at neuromuscular junctions of single muscle fibers are given in Table 2. During drug application, only 50% of the fibers elicited an AP. These APs showed a 41% decrease in OS, a 14% decrease in MRR, and a 26% decrease in MRF from control values. In the remaining fibers, 10% showed no EPPs and 40% gave subliminal EPPs. A typical record of a subliminal EPP from a THC-treated preparation is shown in Fig. 1. In many records an AP was found superimposed on the falling phase of a subliminal EPP, as shown in Fig. 2. We assume that in such records, the bath-applied THC

TABLE 1
Effects of 30 μ M THC on neurally initiated muscle fiber action potentials recorded at nonjunctional regions

Bath sequence	N ^a	RP	AP	OS	MRR	MRF
		-mV	mV	mV	V/s	V/s
Ringer solution	60	90.4 $\pm$ 2.9	127.9 $\pm$ 4.2	37.8 $\pm$ 2.8	487 $\pm$ 22	132 $\pm$ 9
Ringer + 30 μ M THC (10–180 min)	58	89.6 $\pm$ 3.5	126.5 $\pm$ 7.5	34.3 $\pm$ 4.2	404 $\pm$ 46	102 $\pm$ 11
	37 ^b	89.9 $\pm$ 3.1	—	—	—	—
Ringer solution (180–360 min)	54	90.3 $\pm$ 4.7	120.7 $\pm$ 8.2	34.5 $\pm$ 6.5	397 $\pm$ 33	107 $\pm$ 13
	44 ^b	91.5 $\pm$ 5.7	—	—	—	—

^a Number of fibers.

^b No propagated AP.

blocked transmission at the surface junction from which the recording was made and that a propagated AP was initiated in the same fiber at a second junction which was less accessible to the bath-applied THC.

From 16 fibers of muscles bathed in THC (Table 2), average results are as follows: EPP amplitude = 22.1 $\pm$ 7.4 mV, onset to peak time = 1.57 $\pm$ 0.37 ms, and peak to $\frac{1}{2}$ decay time = 4.25 $\pm$ 1.21 ms (11 fibers). From muscles treated with THC for 180 min and then returned to Ringer solution, average results for 19 fibers are as follows: EPP amplitude = 15.3 $\pm$ 9.4 mV, onset to peak time = 1.6 $\pm$ 0.33 ms, and peak to $\frac{1}{2}$ decay time = 4.68 $\pm$ 1.64 ms (13 fibers). The EPPs in THC-treated preparations have a slow onset and a long decay time in comparison with EPPs recorded from preparations blocked by *d*-tubocurarine (9).

In addition to its action on EPPs, THC caused changes in the amplitude and frequency of spontaneous MEPPs. Referring to Table 2, it can be seen that for 21 THC-treated junctions, the MEPP amplitude increased from a control value of 0.67 to 0.95 mV and the frequency fell from 24 to 19/min. For 16 THC-treated junctions at which the EPP amplitude was subliminal and no AP was initiated, the MEPP amplitude was still greater, having a value of 1.1 mV, and the frequency was further diminished to 11/min. Finally, for 4 junctions at which neural stimulation evoked no EPP whatsoever, the MEPPs reached the greatest amplitude and the lowest frequency. It appears that the more intense the neuromuscular block, judging from the EPP amplitude, the greater the MEPP amplitude and the lower the frequency. We suppose that this sequence results from a progressive loading of the preparation with THC.

After removal of the THC-containing bath and its replacement with Ringer solution, ethanol taken up by the preparation is rapidly removed since its effects are known to be quickly reversible. Nevertheless, the neuromuscular transmission block persisted in many fibers. The elevation in MEPP amplitude and the reduction in frequency were greatest for fibers in which the EPP was subliminal. These changes can be attributed to THC which was retained by the preparation and continued to exert a powerful effect for 3 h after the removal of THC from the bathing solution.

Control experiments were conducted on preparations equilibrated in 1% ethanol-containing Ringer solution. Recordings from nonjunctional and junctional regions of 12 muscle fibers taken before, during, and after ethanol application showed no significant changes in AP characteristics. However, the MEPP amplitude was increased 24% from the control value of 0.54 $\pm$ 0.15 mV, to 0.67 $\pm$ 0.12 mV, and the MEPP frequency was increased 82%, from 23 $\pm$ 6 to 42 $\pm$ 2/min. These effects of ethanol were reversible.

A record of a high-amplitude-low-frequency MEPP can be seen in Fig. 3 (center). This MEPP is 3.3 mV in amplitude; the onset to peak time is 6.5 ms. On the left is an ordinary MEPP with 1-mV amplitude and 2.6-ms onset to peak time. We suppose that the high-amplitude, slow-rising MEPPs seen in THC-treated preparations (and to a lesser extent in ethanol-treated preparations) might be produced by a more or less synchronous release of several quanta of ACH. This speculation is based on the fact that large MEPPs sometimes had a waveform that appeared to result from superimposed ordinary MEPPs.

TABLE 2
Effects of 30 μ M THC on neurally initiated muscle fiber action potentials recorded at the neuromuscular junction

Bath sequence	N ^a	RP	AP	OS	MRR	MRF	MEPP	
		-mV	mV	mV	V/s	V/s	Ampl. mV	Freq. min ⁻¹
Ringer solution	46	90.7 $\pm$ 2.1	124.5 $\pm$ 5	33.3 $\pm$ 3	454 $\pm$ 16	121 $\pm$ 15	0.67 $\pm$ 0.4	24.8 $\pm$ 3.1
Ringer + 30 μ M THC (10–180 min)	21	86.2 $\pm$ 5.4	119.4 $\pm$ 8	19.5 $\pm$ 4	392 $\pm$ 22	89 $\pm$ 21	0.95 $\pm$ 0.2	19.2 $\pm$ 6.3
	16 ^b	87.7 $\pm$ 3.7	—	—	—	—	1.1 $\pm$ 0.3	11.0 $\pm$ 8.0
	4 ^b	91.3 $\pm$ 1.8	—	—	—	—	1.2 $\pm$ 0.2	3.8 $\pm$ 1.5
Ringer solution (180–360 min)	23	90.8 $\pm$ 3.4	116.4 $\pm$ 7	25.9 $\pm$ 7	397 $\pm$ 18	100 $\pm$ 17	0.84 $\pm$ 0.3	8.4 $\pm$ 2.7
	19 ^b	93.0 $\pm$ 5.2	—	—	—	—	1.2 $\pm$ 0.8	5.4 $\pm$ 3.1
	10 ^b	92.3 $\pm$ 2.1	—	—	—	—	0	0

^a Number of fibers.

^b No propagated AP.

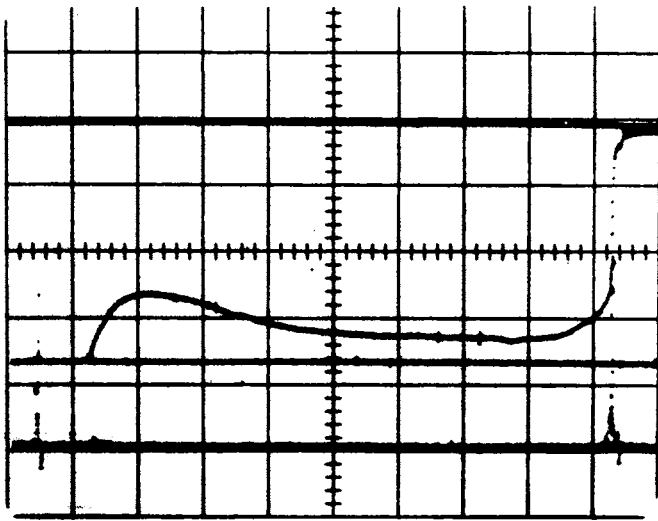


FIG. 1. End-plate potential (EPP) recorded from frog sartorius muscle in Ringer solution containing $30 \mu\text{M}$ THC

EPP amplitude = 25 mV, onset to peak time = 1.8 ms, peak to $\frac{1}{2}$ decay = 5.2 ms. Y calibration = 25 mV/div, X calibration = 2 ms/div.

Additional explanations for the increased MEPP amplitude are that THC causes (a) an increase in the PJM sensitivity, (b) an increase in the membrane input resistance, (c) a decrease in the membrane time constant, and (d) a decrease in the ACH esterase activity. These possibilities, with the exception of d, were tested in the following experiments. Hoekman *et al.* (4) have found no significant change in cholinesterase activity in rat phrenic nerve–diaphragm preparations treated with THC.

Effects of THC on the sensitivity of the PJM to applied CARB. The sensitivity of the PJM to applied CARB was determined by recording the depolarization produced at the junctional region during CARB perfusion. Results given in Table 3 indicate that during 1–3 h in THC, the

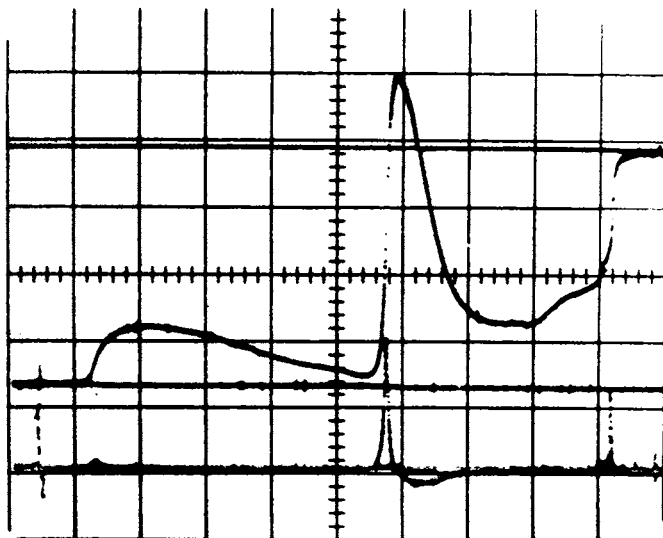


FIG. 2. End-plate potential (EPP) followed by an action potential (AP) recorded from frog sartorius muscle in Ringer solution containing $30 \mu\text{M}$ THC

Y calibration: upper trace = 25 mV/div; lower trace = dV_m/dt , 250 mV/ms/div. X calibration = 2 ms/div.

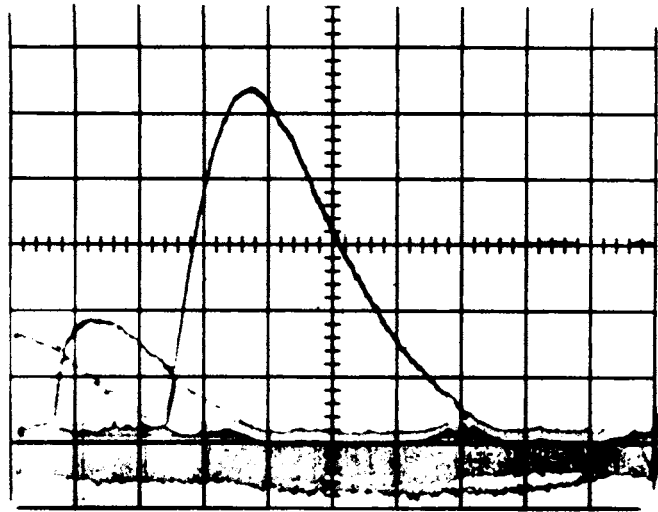


FIG. 3. Miniature end-plate potentials (MEPPs) recorded from frog sartorius muscle in Ringer solution containing $30 \mu\text{M}$ THC

Center: high-amplitude-low-frequency MEPP, 3.3-mV amplitude, onset to peak time = 6.5 ms. Left: ordinary MEPP, 1-mV amplitude, onset to peak time = 2.6 ms. Y calibration = 0.625 mV/div, X calibration = 5 ms/div.

PJM sensitivity increases, judging from the fact that depolarization produced by applied CARB shows a significant increase of 21.5%, from a control value of 19.5 ± 5.2 to 23.7 ± 3.5 mV. A small part of the increase in PJM sensitivity appears to be produced by ethanol alone, but our data are insufficient to prove this statistically. Evidently, the increase in PJM sensitivity as measured by CARB perfusion cannot account for the increase in MEPP amplitude produced by the application of THC (Table 2).

Effects of THC on the membrane input resistance and membrane time constant. The membrane input resistance and time constant were measured at the junctional region. Thereafter the nerve was stimulated and the resulting membrane activity was determined. Results given in Table 4 indicate, for fibers in which the EPP was liminal, that no significant changes occur in the input resistance or time constant during THC treatment or after the removal of THC from the bath. However, in fibers where subliminal EPPs were recorded, a decrease in the time constant was observed.

TABLE 3
Postjunctional membrane sensitivity tested with $20 \mu\text{M}$ carbamylcholine perfusion

Bath	N ^a	RP -mV	Depolarization mV
Ringer solution	12	87.8 ± 3.3	19.5 ± 2.2
Ringer + $30 \mu\text{M}$ THC (10–60 min)	10	91.2 ± 2.0	21.2 ± 4.9
Ringer + $30 \mu\text{M}$ THC (60–180 min)	9	86.0 ± 4.4	$23.7 \pm 3.5^*$
Ringer solution (180–360 min)	8	91.9 ± 3.4	21.3 ± 5.4
Ringer + 1% ethanol (10–120 min)	12	90.1 ± 3.3	21.0 ± 3.3

^a Number of junctions.

* $P < 0.01$.

TABLE 4

Effects of 30 μ M THC on membrane input resistance and membrane time constant at the neuromuscular junction

Bath sequence	N ^a	RP	R _{in}	t
		-mV	Meg	ms
Ringer solution	12	93.4 $\pm$ 4.1	0.63 $\pm$ 0.22	15.0 $\pm$ 1.8
Ringer + 30 μ M THC (10-180 min)	10	93.1 $\pm$ 3.0	0.53 $\pm$ 0.11	15.5 $\pm$ 3.2
Ringer solution (180-360 min)	9	94.4 $\pm$ 3.3	0.62 $\pm$ 0.18	15.0 $\pm$ 1.4
	4 ^b	96.3 $\pm$ 0.5	0.53 $\pm$ 0.16	11.6 $\pm$ 1.7

^a Number of junctions.

^b Subliminal EPPs were recorded in these fibers.

Effects of THC on the quantal content. The data so far described have shown that THC causes an increase in MEPP amplitude and a small increase in PJM sensitivity but, nonetheless, following its application a single nerve impulse often produces a subliminal EPP. Thus one must consider possible presynaptic sites of action to explain the block of neuromuscular transmission by THC. To test this, THC was applied and the release of ACH per nerve impulse was determined by measuring the quantal content.

Table 5 shows the effect of THC on quantal content. All junctions tested showed a decrease in quantal content 20 min after THC was applied. This marked effect of THC becomes further intensified with time. The slight increase in quantal content which occasionally appears early after the cessation of drug perfusion we presume to be due to the action of ethanol.

DISCUSSION

The purpose of the present study was to provide evidence as to the sites and mechanisms of action of THC. We found that THC acts on conductile membranes of muscle fibers, where it reduced the amplitude and rates of rise and fall of muscle APs. Since the rising phase of

an AP is associated principally with an increase in sodium conductance and the falling phase with an increase in potassium conductance, we conclude that THC depresses both of these conductances.

THC also exerts a powerful blocking action on neuromuscular transmission. It diminishes both the AP evoked and the spontaneous release of ACH from neuronal terminals. The evidence for this is that THC caused the EPP amplitude to be greatly decreased and it also diminished the spontaneous MEPP frequency. The neuromuscular transmission block produced by THC provides an explanation for the muscle weakness experienced by *Cannabis* smokers. Hollister *et al.* (10) reported many years ago that one of the acute effects of *Cannabis* smoking was a decrease in muscle strength as measured with a finger ergograph. Ptosis of the eyelids and impairment of body and hand steadiness were observed by Domino (11).

We have found that during application of THC and following its removal from the bathing solution, MEPPs increase in amplitude. A number of mechanisms could have been responsible for this increase. The first is that high-amplitude MEPPs are produced by the rapid release of two or more ACH quanta from a single presynaptic release site. Some of the MEPP waveforms we recorded show evidence of such summation.

The second possible factor which could increase MEPP amplitude is that a change in the passive electrical properties of the PJM and surrounding membrane is produced by THC. However, both the input resistance and the membrane time constant were unchanged during THC application and following the return of the preparation to Ringer solution (Table 4). During the latter period a few fibers showed a reduced time constant, but the data are limited and their significance cannot be assessed without further exploration.

A third factor which contributes to the increase in MEPP amplitude is a THC-produced increase in PJM sensitivity to CARB. However, the increase in PJM sensitivity which we measured is too small to provide a complete explanation. We measured PJM sensitivity by perfusing carbamylcholine onto junctions of THC-treated preparations and the test requires 30-60 s for its completion. Thus if the THC and ethanol accelerate receptor desensitization, the PJM sensitivity could have been underestimated. We attempted to measure PJM sensitivity by the use of iontophoretic techniques. This procedure was not satisfactory because after THC treatment it was difficult to locate responsive spots on the PJM. An investigation of the possible effect of THC on receptor desensitization is needed and we intend to pursue this problem.

The work of other investigators (13, 14) has shown that 1% ethanol can increase the amplitude and frequency of MEPPs, the amplitude of iontophoretically produced ACH potentials, and the amplitude of EPPs recorded during *d*-tubocurarine block. Thus in our experiments we faced the problem of differentiating between the effects of ethanol and THC on MEPP amplitude. The hydrophobic character of THC creates a difficulty when one wishes to apply this drug as a constituent of aqueous media. THC was supplied to us as a 3 mM

TABLE 5

Effects of 30 μ M THC on quantal content

Recording period	N ^a	Average quantal content
<i>min</i>		<i>m</i>
Control	2	3.1
Post-THC 0-10		0.8
Control	3	3.2
Post-THC 0-10		1.2
10-15		0
Control	2	3.5
Post-THC 0-20		3.9
20-30		1.2
Control	3	2.4
Post-THC 0-15		1.8
15-20		0.5
20-30		0
Control	4	2.8
Post-THC 0-10		0.8
10-20		0.3
20-30		0
Control	1	1.5
Post-THC 0-30		1.7
30-60		1.4

^a Number of junctions.

solution in absolute ethanol; hence a 30 μ M test solution of THC made from this stock unavoidably contains 1% (0.17 M) ethanol.

In our study, in order to estimate any contributing effects of ethanol, control experiments were carried out using THC-free Ringer solution containing 1% ethanol. In addition, we differentiated between effects produced by ethanol and those caused by THC by taking advantage of the fact that the effects of ethanol are rapidly reversible (12–14), but the removal of THC occurs relatively slowly. Thus the neuromuscular block and the high-amplitude MEPPs which persisted when the test solution (THC plus ethanol) was replaced by Ringer solution can be ascribed to THC. One must also bear in mind that ethanol, in contradistinction to THC, *increases* MEPP frequency and it also potentiates neuromuscular transmission. Evidently the presynaptic effect of THC in reducing spontaneous quantal release and quantal content can override the potentiating action of ethanol.

We found that during the application of THC the release of ACH per nerve impulse decreased continuously and sometimes ceased altogether. These results are correlated with the THC-produced depression in twitch response recorded in guinea pig ileum (1, 2) and with the slow-developing blockade to indirect stimulation observed in rat phrenic nerve–diaphragm preparations (3). The observation that THC reduces the amplitude of muscle fiber APs provides a possible explanation for its capacity to decrease the presynaptic release of ACH. THC might also act by reducing the calcium influx associated with AP propagation in neuronal terminals.

We have found that EPPs recorded during and following the application of THC have a prolonged time course compared with those recorded from preparations blocked with *d*-tubocurarine. This change (which can also be caused by ethanol (15)) can be ascribed to THC because it persists after the return of the preparation to Ringer solution, at which time the action of ethanol is reversed (15). The mechanism by which THC prolongs the time course of EPPs is unknown at present. One possibility is

that it increases the opening time of ACH-activated ionic channels of the PJM. Noise analysis is needed to provide evidence bearing on this speculation.

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Send reprint requests to: William L. Nastuk, Department of Physiology, College of Physicians and Surgeons, Columbia University, New York, New York 10032.