NOVEL ACTION OF A PIPERAZINE DERIVATIVE ON THE END-PLATE OF THE FROG

Kenji KUBA, Kenichi CHIKAZAWA, and Kyozo KOKETSU

*Department of Physiology,
Kurume University, School of Medicine,
Kurume 830, Japan*

Abstract The effects of a piperazine derivative, trimetazidine (1-(2, 3, 4-trimethoxybenzyl) piperazine dihydrochloride) on the frog end-plate membrane were studied. Action and resting membrane potentials and the input resistance of muscle fibers were not affected by trimetazidine (82–165 μM). Under these conditions, the frequency of the miniature end-plate potentials was unchanged while its amplitude was slightly decreased. The amplitude of acetylcholine (ACh) potentials were markedly and reversibly decreased after application of trimetazidine (82–165 μM). The dose response curve of the end-plate membrane to ACh showed a non-competitive type of blockade. Trimetazidine (165 μM) not only decreased the amplitude of the end-plate currents (EPC) recorded from the glycerinated muscles using a voltage clamp technique, but also drastically shortened its time course. Under these conditions, the falling phase of the EPC became completely voltage insensitive. The equilibrium potential for the EPC slightly shifted to a more negative value in the presence of trimetazidine (165 μM). Coefficient of variation of EPC was increased by trimetazidine (165 μM), indicating a decrease in the quantal content of the EPC. The rate of desensitization of the end-plate to ACh was facilitated and the rate of decrease in EPC amplitude during tetanic stimulation became voltage sensitive by the action of trimetazidine (133 μM). It is concluded that trimetazidine mainly acts on the postsynaptic membrane with a weak presynaptic action. The agent seems to block a step subsequent to the interaction of ACh with its receptor, which presumably involves changes in the ion conductance of the membrane and is responsible for the voltage sensitivity of the response.

It has been reported by many workers that under physiological conditions, the decay phase of end-plate currents (EPC), obtained by voltage-clamping of the end-plate membrane, follows almost a single exponential function (TAKEUCHI and TAKEUCHI, 1959; OOMURA and TOMITA, 1960; MAENO *et al.*, 1971; KORDAS, 1972a,

Received for publication December 8, 1975

久場健司, 近沢健一, 額額教三

b; MAGLEBY and STEVENS, 1972a; KUBA *et al.*, 1973, 1974). This implies that one of a series of steps, following release of acetylcholine (ACh) up to the conductance increase at the end-plate membrane, is a rate limiting step for the decay of the EPC. Not only the time course of transmitter release (KATZ and MILEDI, 1965), but also the life time of ACh (*cf.* MAGLEBY and STEVENS, 1972b; KUBA *et al.*, 1973), appears to be rather short in comparison with the EPC time course. Therefore, the most likely step responsible for the exponential decay of the EPC could be dissociation of ACh from its receptor (KORDAS, 1972a) or the process directly involved in the conductance change of the end-plate membrane (MAGLEBY and STEVENS, 1972b; KUBA *et al.*, 1973). In any case, the rate-determining step for the EPC decay seems to be voltage sensitive; the greater the negativity of membrane potential the longer was the decay of the EPC (KORDAS, 1969 and 1972b; MAGLEBY and STEVENS, 1972a; KUBA *et al.*, 1973; ALBUQUERQUE *et al.*, 1974; KUBA *et al.*, 1974).

Histrionicotoxin, which was isolated from the Colombian arrow-poison frog, *Dendrobates histrionicus* (DALY *et al.*, 1971), was found to block a conductance increase of the end-plate membrane by ACh without affecting the binding of ACh to the receptor (ALBUQUERQUE *et al.*, 1973a, b, and 1974). This toxin, a spiro-piperidine alkaloid, drastically modified the voltage sensitivity of the EPC (ALBUQUERQUE *et al.*, 1974). The main component of venom from the fire ant, *Solenopsis*, which is a piperidine derivative, seems to have similar actions on the end-plate membrane (YEH *et al.*, 1975). It has been reported that a derivative of another heterocyclic amine, trimetazidine (See Fig. 1 for molecular structure), also blocks the response of the subsynaptic membrane to ACh in the skeletal muscles and sympathetic ganglion cells of frogs (MINOTA and KOKETSU, 1975). In this investigation, the mechanism of its action on the end-plate membrane was analyzed in the hope that this agent could be used as a tool to elucidate mechanisms of the chemical excitation of the end-plate membrane. This agent was found to have novel effects on the time course of the EPC and its voltage dependency. Analysis led to the conclusion that trimetazidine acts on the process directly involved in the conductance change of the end-plate membrane.

METHODS

Nerve-muscle preparations of the sartorius muscles were dissected from frogs (*Rana nigromaculata*) in most experiments. The extensor longus digitorum IV muscles of bullfrogs (*Rana catesbeiana*) were sometime used in experiments in which surface potential changes of the end-plate to ACh were recorded. The ionic composition of Ringer's solution was as follows: NaCl, 115; KCl, 2.0; CaCl₂, 1.8; Na₂HPO₄, 1.3; NaH₂PO₄, 0.7 (in mM). In the experiments to record miniature end-plate potentials (MEPP), the concentration of KCl was increased to 10 mM and its tonicity was adjusted by reducing NaCl to an equimolar amount. Neostigmine (2 μ M) was added to the perfusing solution in order to increase the amplitude of MEPP. All the experiments were carried out at room temperature (19–23°C).

The sartorius muscles used for recording EPCs and action potentials were treated with 400 mM glycerol Ringer's solution as originally described by FUJINO *et al.* (1961) and established by GAGE and EISENBERG (1967). The muscles were perfused with the Ringer's solution containing glycerol 400 mM for one hour and returned to normal Ringer's solution. Although resting membrane potentials of the muscle fibers fell to relatively low values (-65 mV to -90 mV), this procedure completely eliminated the contraction of the muscle.

A conventional technique was used to record intracellular potential changes of the muscle fibers. Microelectrodes were filled with 3 M KCl, and those having tip resistance of 5–20 M Ω were selected for recording intracellular potentials. The circuit for voltage clamp experiments was essentially similar to those described by TAKEUCHI and TAKEUCHI (1959) and OOMURA and TOMITA (1960). The feedback amplifier used was a high voltage output operational amplifier (Burr Brown 3460). EPCs were monitored with another operational amplifier (Burr Brown 3401) which was connected so as to convert current to voltage. Electrodes used for current injection were filled with 3 M KCl and had tip resistances less than 5 M Ω . Criteria for successful voltage clamping were; 1) that the amplitude of residual end-plate potentials during voltage clamping be less than a few mV in the normal Ringer's solution and 2) that the fastest rise time (less than 0.8 msec at the membrane potential of -90 mV) and the greatest amplitude of the EPCs which be obtainable at each penetrated end-plate.

The dose-response curve of the end-plate to ACh was obtained by recording surface potential changes of the muscle fibers using the fluid electrode technique of FATT (1950). The preparation was washed five to seven times with normal Ringer's solution between each recordings of membrane depolarizations evoked by various concentrations of ACh. When the ACh concentration was successively increased without washing, all the recordings of the responses to different concentrations of ACh were carried out within a period during which desensitization of the receptor was small.

Acetylcholine potentials (ACh potentials) were evoked by iontophoretic application of ACh (2 M) through a microelectrode which had tip resistance of 60–100 M Ω .

Relative changes in quantal contents of the EPCs were calculated by the variance method (DEL CASTILLO and KATZ, 1954; ELMQVIST and QUASTEL, 1965). The variance method which is based on the Poisson theorem would cause overestimation of quantal contents when quantal contents are high (MIYAMOTO, 1975). However, since the purpose of the present study is concerned only with obtaining relative changes in quantal contents of the EPC in the presence of the drug, the use of the variance method may be justified, for a decrease in the coefficient of variation would also indicate an increase in the quantal contents even in case of binomial statistics as shown in Fig. 3 of MIYAMOTO (1975). Series of 30–50 EPCs were evoked by repetitive nerve stimulations (50 Hz) in the normal Ringer's solution and

in solutions containing the drug. The initial potentiation in the amplitude of the EPC was followed by a progressive decline up to 15–20 numbers of the EPC. The decline phases were rather longer in the presence of the drug, particularly at hyperpolarized levels. However, further continuation of stimulation resulted in a steady level of the EPC amplitude with small fluctuations (See Fig. 9B). Therefore, the coefficient of variation of the EPC amplitude was calculated from the last twenty EPCs of a series of EPCs recorded at depolarized levels.

Most of the recordings were displayed on a cathode ray oscilloscope (Nihon Kohden, VC-7). In the experiments for the ACh dose-response curves and in some experiments to record ACh potentials, the responses were recorded on a pen-writing recorder (Nihon Kohden RJG-3022), having frequency characteristics of DC–80 Hz.

Drugs used were trimetazidine hydrochloride (1-(2, 3, 4-trimethoxybenzyl) piperazine hydrochloride), which was a generous gift of Toaeiyo-Kagaku Co., acetylcholine chloride (Wako Pure Chemical Ind.), D-tubocurarine chloride (Wako Pure Chemical Ind.) and neostigmine methylsulfate (Wako Pure Chemical Ind.).

RESULTS

Effects of trimetazidine on the end-plate membrane

Figure 1 shows effects of trimetazidine on ACh potentials evoked by ionto-

Fig. 1. Effect of trimetazidine (82 μ M) on the ACh potentials evoked by iontophoretic application of ACh (2 M). Recordings in B follow those in A. Upper rows indicate membrane potential, while lower rows are currents passed to the ACh electrode. The duration of current pulses was 20 msec. Molecular structure of trimetazidine is shown on the top.

Fig. 2. Lack of effect of trimetazidine (165 μ M) on the action potential and input resistance of the muscle fiber. Recordings in the presence of trimetazidine were taken 5 min after application of the agent. A; action potentials induced by nerve stimulation. Upper, middle and lower beams indicate zero level for membrane potential, membrane potential and the first derivative of the potential change. B: Electrotonic potentials. Upper beams are the current passed to the membrane, while lower beams are membrane potential.

phoretic application. The amplitude of ACh potentials decreased to 25% of the control in the presence of trimetazidine (82 μ M). At a higher concentration of the drug (165 μ M), it decreased to 9% of the control. Recovery from the decrease in amplitude of the ACh potentials was completed within a few minutes after rinsing the muscle with normal Ringer's solution. Resting membrane potentials of muscle fibers remained unchanged, as shown in Fig. 1 (Control; 87.0 ± 1.4 (s. e.) mV, trimetazidine (165 μ M); 85.9 ± 1.3 mV, $n=7$). Lack of effects of trimetazidine (165 μ M) on the resting membrane potential was also observed in glycerol-treated muscle fibers (Control; 83.9 ± 1.4 mV, trimetazidine; 82.3 ± 1.3 mV, $n=7$).

Figure 2 shows effects of trimetazidine (165 μ M) on indirectly elicited action potentials and on the input resistance of muscle fibers pretreated with glycerol. All the parameters, such as the peak amplitude and the maximum rates of rise and fall, of an action potential were not affected by treatment with the drug. The input resistance of the muscle fiber was also unchanged after perfusion of preparations with the solution containing trimetazidine (165 μ M), as shown in Fig. 2.

Effects on transmitter release

Trimetazidine (82 μ M) did not show any significant effects on the frequency of miniature end-plate potentials (MEPP). On the other hand, the amplitude of

Table 1. Coefficient of variation of the EPC.

Cell No.	Control	Trimetazidine	'ratio'	Em
1	1.42×10^{-3}	1.67×10^{-3}	1.18	+34 mV
2	2.64	3.66	1.39	+36
3	3.52	9.35	2.66	-50
4	0.70	1.08	1.54	-50
5	0.87	1.86	2.14	-80
6	0.99	1.14	1.15	-60
Mean $\pm$ s.e.			1.68 $\pm$ 0.22	

The concentration of trimetazidine was 165 μ M. 'Ratio' is calculated by dividing the coefficient of variation of the EPC in the presence of the drug by that of the control value, so that it indicates a relative change in quantal contents (see METHODS). Em is the membrane potential at which the EPCs were measured.

MEPP was slightly decreased in the presence of trimetazidine (82 μ M). For instance, in a fiber perfused with the high K^+ (10 mM) solution containing neostigmine (2 μ M), trimetazidine (82 μ M) decreased the amplitude of the MEPP from 0.92 ± 0.02 mV ($n=90$) to 0.66 ± 0.02 mV ($n=82$), while the frequency of the MEPP remained unchanged (Control; 9.0/sec, trimetazidine; 8.2/sec). Similar results were obtained with four other fibers.

Coefficient of variation of the EPCs, which were recorded from glycerinated fibers by applying repetitive nerve stimulation (50 Hz), as shown in Fig. 9 (see METHODS), was increased in the presence of trimetazidine (165 μ M). The mean in-

Fig. 3. Effects of trimetazidine (33, 133 μ M) and D-tubocurarine (1 μ M) on the dose response curve of the extensor longus digitorum IV muscle to ACh. The ordinate indicate the membrane depolarization recorded by the air-liquid interface electrode. The abscissa is the concentration of ACh. Arrows represent half maximal points.

crease was 68% (Table 1). This result would suggest that quantal content of the EPC was decreased under this condition.

Noncompetitive blocking action of trimetazidine on the end-plate

Figure 3 shows effects of trimetazidine and D-tubocurarine on dose-response curves of the extensor longus digitorum IV muscle to ACh. The membrane depolarization induced by various concentrations of ACh was recorded with an air-liquid interface method (FATT, 1950). The maximum membrane depolarization was markedly suppressed by application of trimetazidine (33 μ M, 133 μ M), while the ACh concentration which gives the half maximum response seems to be unchanged, indicating that overall dissociation constant of the receptor for ACh remained unchanged. On the other hand, D-tubocurarine (1 μ M) exhibited a pure competitive type of blocking action on the ACh response (see also JENKINSON, 1960). These results clearly indicate that the agent acts on a site other than that where ACh binds.

Effects on end-plate currents and its equilibrium potential

Figure 4 shows EPCs recorded from glycerol-treated muscle fibers at different levels of membrane potential. The amplitude of EPCs was decreased to 60% of the control EPC in the presence of trimetazidine (165 μ M). The suppressing effect of the drug on EPCs was much smaller than that observed on the depolarization induced by the bath application of ACh (see Fig. 3) or on the ACh potential (see Fig. 1). The falling phase of the EPC was markedly shortened by treatment of

Fig. 4. The EPCs recorded at different levels of membrane potential before and 5 min after application of trimetazidine (165 μ M). The membrane potential, indicated at the left, was changed by applying voltage pulses (20 msec in duration). Bottom traces shows the clamped membrane potential at -140 mV.

Fig. 5. The relationships between the amplitude of the EPC and membrane potential, in the presence and absence of trimetazidine ($165 \mu\text{M}$). The abscissa represents membrane potential. The ordinate indicates the EPC amplitude. Inward and outward currents were taken as negative and positive, respectively.

preparations with the agent. The effect on the EPC decay seemed to be greater, as the membrane potential was made more negative. Greater suppressions of the

Table 2. Effect of trimetazidine ($165 \mu\text{M}$) on the equilibrium potential of the EPC.

Cell No.	Control	Trimetazidine	Wash	Differences (Cont. -trimet.)	(Trimet. -wash.)
1	0 mV	-9.0mV		-9.0mV	
2	-3.0	-12.0	-7.5mV	-9.0	-4.5mV
3	-7.5	-12.0	-8.0	-4.5	-4.0
4	-3.5	-7.5	-3.5	-4.0	-4.0
5	+3.5	+5.0		+1.5	
6	+4.5	+1.0	+4.5	-3.5	-3.5
7	+12.5	+8.0		-4.5	
8	-2.0	-1.0	0	+1.0	-1.0
9	+3.5	-0.5	+1.0	-4.0	-1.5
10	+11.5	+4.5	+4.5	-7.0	0
	+2.0mV	-2.4mV	-1.3mV	-4.3	-2.6
				$\pm 0.8^* \text{mV}$ (S.E.)	$\pm 0.6^* \text{mV}$

Measurements of the equilibrium potentials were carried out in the same fiber, before, during and after application of the drug. The muscles were equilibrated at least for 5 min in the solution containing trimetazidine and in the normal Ringer's solution after removal of the agent. Numbers indicated with stars are statistically significant from the zero ($P < 0.01$).

amplitudes of ACh potentials and depolarization induced by bath application of ACh could be explained by the same mechanism that produced a shortening of the EPC decay, because a decrease in the EPC decay time would imply a decrease in the life time of opened ion channels, as will be discussed later, which should result in a decrease of a steady membrane depolarization (KATZ and MILEDI, 1972).

Figure 5 shows the relationships between the amplitude of EPCs and membrane potentials in the presence and absence of trimetazidine ($165 \mu\text{M}$). The equilibrium potential for EPCs slightly shifted to a more negative value when the muscle was perfused with a solution containing trimetazidine. Changes in the equilibrium potential observed in ten fibers were summarized in Table 2.

Disappearance of voltage sensitivity of the time course of the EPC

Figure 6 shows logarithmic plots of the falling phase of EPCs recorded at

Fig. 6. The logarithmic plots of the falling phase of EPCs recorded at different levels of membrane potential, before and during treatment of the muscle with trimetazidine ($165 \mu\text{M}$).

different levels of membrane potential (open symbols). The decay of EPCs under normal conditions followed a single exponential function and its slope depended upon the membrane potential, *i. e.*, it was steeper as the negativity of the potential increased. As shown in Fig. 7 (open circles), the logarithm of the half decay time (HDT) of EPC was linearly related to membrane potential. These results are in agreement with previous studies (MAGLEBY and STEVENS, 1972a; KUBA *et al.*, 1973; ALBUQUERQUE *et al.*, 1974). Most of the EPC decay in the presence of trimetazidine ($165 \mu\text{M}$) also followed a single exponential function, although the beginning of the EPC decay deviated from the function (closed symbols in Fig. 6). The slope of its decay under this condition was almost the same at all the levels of membrane potential tested (closed circles in Fig. 7), but sometimes it became slightly steeper at a more negative level of membrane potential. The rise time (RT) of EPCs also depended upon membrane potential in normal Ringer's solution in a manner

Fig. 7. The relationships between the rise and half decay times of the EPC and the membrane potential, in the presence and absence of trimetazidine (165 μ M). The ordinates are the rise and half decay times in the logarithmic scale, while the abscissa is membrane potential.

Fig. 8. Effects of trimetazidine (133 μ M) on the time course of the membrane depolarization induced by the bath application of ACh (10 μ M). The extensor longus digitorum IV muscle was used and the membrane depolarization was measured by the air-liquid interface electrode method. The ordinate indicates the amount of the depolarization expressed in the percentage of the response 1 min after ACh was applied. The abscissa represents time. The muscle was soaked in the solution containing ACh at the zero time.

similar to HDT. Trimetazidine ($165 \mu\text{M}$) shortened RT and made it less potential dependent.

These results clearly indicate that the rate constants for the changes in the ionic conductance of the end-plate membrane by the action of ACh became independent of the electric field across the membrane.

Potency of trimetazidine influenced by repeated applications of ACh and by membrane potential

Figure 8 shows effects of trimetazidine ($133 \mu\text{M}$) on the time course of the membrane depolarization induced by bath application of ACh ($10 \mu\text{M}$). Membrane depolarization was measured with an air-liquid interphase electrode. The percentage of the depolarization to the response 1 min after the start of ACh perfusion

Fig. 9. Effects of trimetazidine ($165 \mu\text{M}$) on the amplitude of the EPCs evoked by repetitive nerve stimulations (50 Hz for 460 (A) and 660 (B) msec) at different levels of membrane potential in two different cells. The ordinate indicates the amplitude of the EPC shown as percentage of the maximum amplitude. The polarity of the EPC was ignored. The abscissa is the time after the beginning of the nerve stimulation. Open symbols are the relative amplitude of the EPC in the normal Ringer's solution, while closed symbols indicate those in the presence of trimetazidine ($165 \mu\text{M}$).

was plotted against time. The agent significantly facilitated the rate of the so called 'desensitization' process of the end-plate membrane (KATZ and THESLEFF, 1957).

It was shown in previous studies (ALBUQUERQUE *et al.*, 1973a, b; ALBUQUERQUE *et al.*, 1974) that histrionicotoxin also progressively decreased the amplitude of a sequence of ACh potentials or those of EPCs evoked by repetitive nerve stimulation, and that its effect was dependent on the membrane potential. Therefore, in view of some similarities of the actions of trimetazidine on the EPC to this toxin, we studied effects of trimetazidine (165 μ M) on the EPCs generated by tetanic stimulation (50 Hz), at different levels of membrane potential. Figure 9 shows such experiments. The rates of decay of the peak amplitude of each EPC during the course of tetanic stimulation are almost the same at all the membrane potentials tested under the normal condition (open signs), while it was faster, when the membrane was made more negative in the presence of trimetazidine (filled signs). This increase in the rate of decline of the EPC peak amplitude, particularly at more negative levels of membrane potential, must be due to the action of the agent on the end-plate membrane, and not to its presynaptic actions.

DISCUSSION

It was shown in the present study that trimetazidine depressed neuromuscular transmission, partially by decreasing the evoked release of transmitter from the nerve terminals, and mainly by inhibiting an increase of the end-plate membrane conductance by transmitter action. The presynaptic action of the drug was indicated by an increase of coefficient of variation of the EPC (Table 1). Evidence for the effects on the end-plate membrane included a marked decrease in the ACh potential amplitude, a decrease in the membrane depolarization induced by ACh perfusion, a slight decrease of MEPP amplitude and a marked shortening of the EPC. Lack of effects of trimetazidine on the ordinary nonsynaptic membrane were shown by no signs of changes in the resting and action potentials and in the input resistance under the effects of the drug. Main interests of the effects of trimetazidine seem to lie in its unique actions on the time course of the EPC. Therefore, these effects will be focused on in this discussion.

There seems to be, at least, two macromolecular units operative in an increase of the membrane permeability to Na^+ and K^+ at the postsynaptic membrane in response to the action of ACh (CHANGEUX *et al.*, 1970). One is the receptor (R) for ACh and the other is customarily called 'ionic channel' or ionic conductance modulator (ICM) which was proposed as a general descriptive designation for the macromolecular entities that are involved in the regulation of ionic conductance of the membrane (KUBA *et al.*, 1973; ALBUQUERQUE *et al.*, 1974; KUBA *et al.*, 1974). Hereafter the customary term 'ionic channel' implying an ionic conductance modulator, will be used. Although it is not known whether these two units are composed of a macromolecule or constitute separate entities, it is clear that they play entirely different roles. These two units may operate in a manner shown in the

following simplified model (KUBA *et al.*, 1973):

where k_1 , k_{-1} , k_2 and k_{-2} are rate constants, and R and R' denote the receptor in its inactive and active conformations, respectively. Io and Ic are the open and closed conformations of the ionic channel, respectively. Although in this model, the cooperativities of the receptor and the ionic channel are not taken into consideration (CHANGEUX *et al.*, 1970; DREYER and PEPPER, 1975), interpretation of the results of the present experiments may not be essentially different whether cooperativity is considered or not. We preferred the kinetic model of the two-step reactions between ACh and receptor proposed by many workers (DEL CASTILLO and KATZ, 1957; STEINBACH, 1968; MAGLEBY and STEVENS, 1972b) to that of the single-step, because of following reasons. It seems to be quite reasonable to assume that the cholinergic receptor would behave like enzymes as suggested (KIRSHNER and STONE, 1951; DEL CASTILLO and KATZ, 1957; MAGLEBY and STEVENS, 1972b), since isolated receptors have been shown to be protein (MILEDI *et al.*, 1971; RAFTERY *et al.*, 1971; MEUNIER *et al.*, 1973). Conformational change of the end-plate membrane was indirectly shown by demonstration of changes in intrinsic birefringence during actions of transmitters (CZILLIK, 1967).

As already stated, either k_{-1} or k_{-2} would determine the decay of the EPC, since it follows almost a single exponential function as shown in Fig. 6 (KORDAS, 1972a; MAGLEBY and STEVENS, 1972b). This suggestion can be supported by the analysis of the electrical noises occurring at the end-plate membrane during actions of ACh (KATZ and MILEDI, 1972; ANDERSON and STEVENS, 1973). MAGLEBY and STEVENS (1972b) suggested that k_{-2} rather than k_{-1} would be a rate-limiting constant for the decay of the EPC on the analogy of the properties of the enzymes. We will tentatively accept this assumption for facilitation of discussion of the present experimental findings. Therefore, any conclusions for k_{-2} can be converted to those for k_{-1} .

Possible mechanisms of drug action. It may be relevant to discuss the site and mechanism of actions of trimetazidine based on this model. There are experimental results to define conditions for the above kinetic schema. First of all, a remarkable suppression of the maximum depolarization indicates a non-competitive type of the inhibitory action of trimetazidine as shown in Fig. 3. It can be inferred, therefore, that this agent acts on a site other than the site where ACh binds. The decrease in the peak amplitude of EPC in the presence of trimetazidine amounted to about 60% of the control. For the quantal contents of EPC seemed to be also decreased under this condition, a decrease in peak amplitude of

the EPC caused by the postsynaptic action of the drug would be much smaller than the decrease actually observed. Accordingly, even under the effects of trimetazidine the ionic channel gate can, at least, be almost fully opened up although its life time may be very short. This means that the above reactions of ACh, receptor and ionic channel should proceed up to the final step of the schema shown above, under this condition. On the other hand, there was a marked shortening of the EPC time course, particularly of its decay, in the presence of the drug. This would imply a greater value for k_{-2} , provided that k_{-2} is much smaller than k_{-1} , or a direct blockade of the open conformation (I_o) of the ionic channel by the drug. An increase in k_{-2} would be caused either by the action of the drug on the ACh·R' or I_o leading to a more rapid conformational change of the ACh·R' or I_o to their inactive forms. Furthermore, a small change in the equilibrium potential for the EPC under the effect of this drug, if it is accepted, might be interpreted as manifestation of the action on the ionic channel. Referring to the results of MAGAZANIK and VYSKOČIL (1969), KATZ and MILEDI (1973) suggested a similar mechanism of the action of atropine on the end-plate membrane. A possibility that trimetazidine increases the affinity of acetylcholinesterase for ACh might explain the shortening of EPC. However, this mechanism is most unlikely because the half decay times of the shortened EPC in the presence of the drug were less than 0.2 msec which is much smaller than the gating time of about 1 msec for the end-plate conductance in the normal Ringer's solution reported by KATZ and MILEDI (1972).

Another interesting effect of the drug is the insensitivity of the EPC decay to membrane potential (Fig. 7). If the shortening of the EPC is explained by an increase in k_{-2} , it means that k_{-2} becomes independent of membrane potential, in another words, the receptor or the ionic channel or both lose their voltage sensitivity in the presence of trimetazidine. If the direct blockade of the I_o by the drug occurs, lack of voltage sensitivity of the EPC decay could simply be a manifestation of the voltage insensitive binding of the drug to the I_o . It is worthy to note that the Hill coefficient of the conductance increase of the electroplaque membrane by agonist, which may measure cooperative interactions among binding sites, do not change with potential and that the voltage sensitivity of the conductance increase were the same for different agonist (LESTER *et al.*, 1975). Thus, if trimetazidine acts on the ionic channel through either of mechanisms mentioned, it may be suggested that the ionic channel is responsible for voltage sensitivity of the EPC decay.

A gradual decrease of the membrane depolarization during the persistent application of ACh to the bathing solution was observed in the presence of trimetazidine (Fig. 8). In addition, a similar progressive suppression of the end-plate conductance was revealed by trains of the EPC's evoked by repetitive nerve stimulation. The rate of this decrease was greater when the membrane was made more negative (Fig. 9). These effects which are very similar to those of histrionicotoxin (ALBUQUERQUE *et al.*, 1974) would be interpreted in two ways. One could be that

trimetazidine facilitates the rate of the desensitization of the postsynaptic membrane which physiologically occurs (KATZ and THESLEFF, 1957). If this is the case, the drug seems to increase the potential sensitive properties of the desensitization process (MAGAZANIK and VYSKOČIL, 1970). However, this explanation might not be consonant with the observation that trimetazidine eliminated the voltage sensitivity of the EPC decay, if it is assumed that both the voltage sensitive properties of the EPC decay and the desensitization process are caused by the same effect of electric field on the receptor or ionic channel. Another explanation would be that the affinity of the drug to the open forms of the receptor or ionic channel may be higher than that to a closed form, so that repeated activation of the receptor and ionic channel results in a progressive blockade of the end-plate conductance as suggested for the effects of histrionicotoxin (ALBUQUERQUE *et al.*, 1974). In this case, the affinity of the agent to the ACh·R' or I_o must be voltage sensitive, indicating different conformational states of the open form of the ACh receptor complex or the ionic channel depending on membrane potential.

It was recently suggested by Gage and his coworkers that the state of membrane lipid may be an important determinant of the kinetics of synaptic conductance change (GAGE *et al.*, 1974, 1975). These authors (GAGE *et al.*, 1974) found that octanol, which is reported to increase the fluidity of the muscle membrane, shortened the time course of the EPC. Therefore, it might be possible that all the effects of trimetazidine observed in this study are explained by the indirect influences on the receptor and/or the ionic channel produced by its direct action on the lipid environment of these units.

In any case, it is quite certain that this agent acts on the mechanism underlying the conductance increase of the end-plate membrane by ACh. This process should involve activated forms of the receptor and ionic channel, and must be responsible for the potential sensitivity of the falling phase of the EPC. Final determination of the site of its action would give an answer to the question whether the receptor or the ionic channel or both is responsible for the voltage sensitive characteristics of the EPC decay.

We thank Toaiei-yokagaku for the gift of trimetazidine hydrochloride, and Miss Akemi Kumamaru for preparation of the manuscript.

REFERENCES

- ALBUQUERQUE, E. X., BARNARD, E. A., CHIU, T. H., LAPA, A. J., DOLLY, J. O., JANSSON, S. -E., DALY, J., and WITKOP, B. (1973a) Acetylcholine receptor and ion conductance modulator sites at the murine neuromuscular junction. *Proc. Nat. Acad. Sci. U.S.A.*, **70**: 949-953.
- ALBUQUERQUE, E. X., KUBA, K., and DALY, J. (1974) Effect of histrionicotoxin on the ionic conductance modulator of the cholinergic receptor: A quantitative analysis of the end-plate current. *J. Pharmacol. Exp. Ther.*, **189**: 513-524.
- ALBUQUERQUE, E. X., LAPA, A. J., KUBA, K., DALY, J., and WITKOP, B. (1973b) A study of the effects of histrionicotoxin on the acetylcholine receptor-ionic conductance modulator complex. *Trans. Am. Soc. Neurochem.*, **4**: 50.

- ANDERSON, C. R. and STEVENS, C. F. (1973) Voltage clamp analysis of acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol. (Lond.)*, **235**: 655-691.
- CHANGEUX, J. -P., PODLESKI, T., KASAI, M., and BLUMENTHAL, R. (1970) Some molecular aspects of membrane excitation studied with the eel electroplax. *In: Excitatory Synaptic Mechanisms*, ed. by ANDERSEN, P. and JANSEN, J. K. S. Universitetsforlaget, Oslo.
- CZILLIK, B. (1967) *In: Functional Structure of the Postsynaptic Membrane in the Myoneural Junction*. Academy Press, Budapest.
- DALY, J. W., KARLE, I., MYERS, C. W., TOKUYAMA, T., WATERS, J. A., and WITKOP, B. (1971) Histrionicotoxins: Roentgen-ray analysis of the novel allenic and acetylenic spiroalkaloids from a Colombian frog, *Dendrobates histrionics*. *Proc. Nat. Acad. Sci. U.S.A.*, **68**: 1870-1875.
- DEL CASTILLO, J. and KATZ, B. (1954) Quantal components of the end-plate potential. *J. Physiol. (Lond.)*, **124**: 560-573.
- DEL CASTILLO, J. and KATZ, B. (1957) Interaction at end-plate receptors between different choline derivatives. *Proc. R. Soc. Lond. B.*, **146**: 369-381.
- DREYER, F. and PEPPER, K. (1975) Density and dose-response curve of acetylcholine receptors in frog neuromuscular junction. *Nature*, **253**: 641-643.
- ELMQVIST, D. and QUASTEL, D. M. J. (1965) A quantitative study of end-plate potentials in isolated human muscle. *J. Physiol. (Lond.)*, **178**: 505-529.
- FATT, P. (1950) The electromotive action of acetylcholine at the motor end-plate. *J. Physiol. (Lond.)*, **111**: 408-422.
- FUJINO, M., YAMAGUCHI, T., and SUZUKI, K. (1961) "Glycerol effect" and the mechanism linking excitation of the plasma membrane with contraction. *Nature (Lond.)*, **192**: 1159-1161.
- GAGE, P. W. and EISENBERG, R. S. (1967) Action potentials without contraction in frog skeletal muscle fibers with disrupted transverse tubules. *Science (Washington)*, **158**: 1702-1703.
- GAGE, P. W., MCBURNEY, R. N., and SCHNEIDER, G. T. (1975) Effects of some aliphatic alcohols on the conductance change caused by a quantum of acetylcholine at the toad end-plate. *J. Physiol. (Lond.)*, **244**: 409-429.
- GAGE, P. W., MCBURNEY, R. N., and VAN HELDEN, D. (1974) End-plate currents are shortened by *octanol*: Possible role of membrane lipid. *Life Sciences*, **14**: 2277-2283.
- JENKINSON, D. H. (1960) The antagonism between tubocurarine and substances which depolarize the motor end-plate. *J. Physiol. (Lond.)*, **152**: 309-324.
- KATZ, B. and MILEDI, R. (1965) The measurement of synaptic delay, and the time course of acetylcholine release at the neuromuscular junction. *Proc. R. Soc. Lond. B.*, **161**: 483-396.
- KATZ, B. and MILEDI, R. (1972) The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol. (Lond.)*, **224**: 665-700.
- KATZ, B. and MILEDI, R. (1973) The effect of atropine on acetylcholine action at the neuromuscular junction. *Proc. R. Soc. Lond. B.*, **184**: 221-226.
- KATZ, B. and THESLEFF, S. (1957) A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *J. Physiol. (Lond.)*, **138**: 63-80.
- KIRSHNER, L. B. and STONE, W. E. (1951) Action of inhibitors at the myoneural junction. *J. Gen. Physiol.*, **34**: 821-834.
- KORDAS, M. (1969) The effect of membrane polarization on the time course of the end-plate current in frog sartorius muscle. *J. Physiol. (Lond.)*, **204**: 493-502.
- KORDAS, M. (1972a) An attempt at an analysis of the factors determining the time course of the end-plate current, I. The effects of prostigmine and of the ratio of Mg^{2+} to Ca^{2+} . *J. Physiol. (Lond.)*, **224**: 317-332.
- KORDAS, M. (1972b) An attempt at an analysis of the factors determining the time course of the end-plate current, II. Temperature. *J. Physiol. (Lond.)*, **224**: 333-348.

- ✓ KUBA, K., ALBUQUERQUE, E. X., and BARNARD, E. A. (1973) Diisofluorophosphate: Suppression of ionic conductance of the cholinergic receptor. *Science* (Washington), **181**: 853-856.
- ✓ KUBA, K., ALBUQUERQUE, E. X., DALY, J. W., and BARNARD, E. A. (1974) A study of the irreversible cholinesterase inhibitor, diisofluorophosphate, on time course of end-plate currents in frog sartorius muscle. *J. Pharmacol. Exp. Ther.*, **189**: 499-512.
- LESTER, H. A., CHANGEUX, J. -P., and SHERIDAN, R. E. (1975) Conductance increases produced by bath application of cholinergic agonists to *Electrophorus* Electroplaques. *J. Gen. Physiol.*, **65**: 797-816.
- MAENO, T., EDWARDS, C., and HASHIMURA, S. (1971) Differences in effects on end-plate potentials between procaine and lidocaine as revealed by voltage-clamp experiments. *J. Neurophysiol.*, **34**: 32-46.
- MAGAZANIK, L. G. and VYSKOČIL, F. (1969) Different action of atropine and some analogues on the end-plate potentials and induced acetylcholine potentials. *Experientia*, **25**: 618-619.
- MAGAZANIK, L. G. and VYSKOČIL, F. (1970) Dependence of acetylcholine desensitization on the membrane potential of frog muscle fibre and on the ionic change in the medium. *J. Physiol.* (Lond.), **210**: 507-518.
- MAGLEBY, K. L. and STEVENS, C. F. (1972a) The effect of voltage on the time course of end-plate currents. *J. Physiol.* (Lond.), **223**: 151-171.
- MAGLEBY, K. L. and STEVENS, C. F. (1972b) A quantitative description of end-plate currents. *J. Physiol.* (Lond.), **223**: 173-197.
- MEUNIER, J. -C., SUGIYAMA, H., CARTAUD, J., SEALOCK, R., and CHANGEUX, J. -P. (1973) Functional properties of the purified cholinergic receptor protein from *Electrophorus electricus*. *Brain Res.*, **62**: 307-315.
- MILEDI, R., MOLINOFF, P., and POTTER, L. T. (1971) Isolation of the cholinergic receptor protein of *Torpedo* electric tissue. *Nature* (Lond.), **229**: 554-557.
- MINOTA, S. and KOKETSU, K. (1975) The actions of trimetazidine on nerve and muscle cells in frogs. *Arch. Int. Pharmacol. Ther.*, **219**: 111-121.
- MIYAMOTO, M. D. (1975) Binomial analysis of quantal transmitter release at glycerol treated frog neuromuscular junctions. *J. Physiol.* (Lond.), **250**: 121-142.
- OOMURA, Y. and TOMITA, T. (1960) Study on properties of neuromuscular junction. In: *Electrical Activity of Single Cells*, ed. by KATSUKI, Y. Igaku-shoin, Tokyo, pp. 181-205.
- RAFTERY, M. A., SCHMIDT, J., CLARK, D. G., and WOLCOTT, R. G. (1971) Demonstration of a specific α -bungarotoxin component in *Electrophorus electricus* electroplax membranes. *Biochem. Biophys. Res. Commun.*, **45**: 1622-1629.
- STEINBACH, A. B. (1968) A kinetic model for the action of xylocaine on receptors for acetylcholine. *J. Gen. Physiol.*, **52**: 162-180.
- TAKEUCHI, A. and TAKEUCHI, N. (1959) Active phase of frog's end-plate potential. *J. Neurophysiol.*, **22**: 395-411.
- YEH, J. Z., NARAHASHI, T., and ALMON, R. R. (1975) Characterization of neuromuscular blocking action of piperidine derivatives. *J. Pharmacol. Exp. Ther.*, **194**: 373-383.