ACTION OF ACETYLCHOLINE ON THE PURKINJE FIBER STUDIED BY VOLTAGE CLAMP TECHNIQUE

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In spite of the general belief that acetylcholine has no effect on the mammalian cardiac ventricle, several reports have appeared describing a slight effect on the action potential of the Purkinje fiber²⁸⁾ and of the ventricular muscle fiber¹⁸⁾ and its inotropic action on the ventricle in a high concentration^{1,2,12,25,29)}. The ionic basis of acetylcholine effects on the atrium was examined to some extent by FOZZARD and SLEATOR¹¹⁾ by the current clamp method. Such being the state, the ionic basis of acetylcholine effects on the Purkinje fibers was studied by the voltage clamp method.

METHOD

Young calf hearts obtained from the slaughterhouse were brought to the laboratory in cool (about 4°C) Tyrode solution. The pH of the Tyrode solution was set at 7.4 by adding an appropriate amount of NaOH. Bundles of Purkinje fibers (diameter 0.3 to 0.5 mm) were removed from the right ventricles. Two ligatures were set at a distance of 1 to 2 mm from each other by means of silk threads as proposed by DECK, KERN and TRAUTWEIN⁴⁾. The preparations were kept in a Tyrode solution of 33°C, saturated with a mixture of 95% O₂ and 5% CO₂. The Tyrode solution was changed to a sodium-free solution when necessary, which was prepared by replacing the 137 mM NaCl of Tyrode solution with 137 mM choline-chloride (choline-Tyrode solution) or 274 mM sucrose (sucrose-Tyrode solution). The composition of these solutions and the buffer followed DUDEL, PEPPER and TRAUTWEIN⁹⁾ in the main.

Membrane voltage was clamped by a device consisting of an intracellular electrode which measures the membrane potential, a second intracellular electrode through which a current is fed into the fiber, and a feedback amplifier which supplies the correct amount of current in order to keep the membrane potential at a chosen level. Other details of the voltage clamp method and apparatus are the same as described by DECK, KERN and TRAUTWEIN⁴⁾. Assuming that the Purkinje fiber bundle of several closely packed cells behave electrically like a single cell, its surface membrane was approximately determined, and these values were used to calculate the current densities (A/cm²) of the feedback clamp current, as described by them.

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RESULTS

The control state

Compared with the results of our experiments with the rabbit and dog heart, the resting potential of some preparations of calf Purkinje fibers was often slightly smaller, being about -60 mV. Preparations which showed a resting potential less than this value were discarded. Since other preparations of calf Purkinje fibers showed a resting potential as large as about -90 mV, this relatively small resting potential was probably due to occasional unfavorable conditions in the slaughterhouse.

The current voltage relation in the control state was similar to that reported by DECK and TRAUTWEIN⁵⁾. When the membrane was depolarized by clamping from -80 mV to a constant level for 300 msec in a stepwise fashion up to -10 mV the curves showed generally the S form, and the

FIG. 1. Voltage-dependent and time-dependent changes of the chord conductance of a calf Purkinje fiber in the control state in depolarization to -10 mV (upper tracings) and to -16 mV (lower tracings).

current at 25 msec after the onset of the clamp was negative at about -20 mV and showed a negative slope between -45 mV to -20 mV.

Assuming that the ionic current of the cell membrane consists chiefly of Na^+ and K^+ , as HODGKIN and HUXLEY^{15,16)} did on the giant axon of *Loligo*, and that the potassium current can be measured when the NaCl of Tyrode solution was replaced by sucrose, the chord conductances for sodium (g_{Na}) and potassium (g_{K}) were calculated. When the current voltage relation was examined under normal conditions only, the NaCl of Tyrode solution was replaced by cholinechloride in some experiments, but no conspicuous difference was observed from the results by replacement with sucrose. The time changes of g_{Na} and g_{K} when the voltage was clamped at -10 mV (the upper figure) and at -16 mV (the lower figure), from the resting potential level of -60 mV are shown in FIG. 1. The g_{Na} , which increased markedly at the beginning of depolarization, decreased rapidly until about 50 msec after the onset of depolarization and then slowly until 300 msec, which was the total duration of the depolarizing step. The g_{K} , which showed a marked decrease

FIG. 2. An example of change of membrane current during depolarization after addition of acetylcholine to Tyrode solution. Resting and clamp potentials of the preparation and duration of depolarization are indicated in FIG. A. The membrane current is shown in FIG. B as follows. B1: in the control state; B2: 1.5 minutes after addition of acetylcholine to a concentration of 10^{-5} g/ml; B3: after 2.5 minutes; B4: after 3 minutes and immediately after further addition of acetylcholine to concentration of 10^{-3} g/ml; B5: after 4 minutes; B6: after 6.5 minutes.

at the beginning of depolarization, showed a fairly rapid increase until 100 msec after the onset of depolarization and a gradual increase thereafter.

Effects of acetylcholine

Acetylcholine was dropped into the solution to obtain the desired final concentration.

Up to the final concentration of 10^{-6} g/ml, no significant change in the ionic currents of Purkinje fibers was noticed, compared with the control. In a concentration higher than 10^{-6} g/ml there were some changes which varied with time. Namely, the measured current decreased initially and increased later. In the experiment shown in FIG. 2 the voltage was clamped at +10 mV from the resting potential of -90 mV and acetylcholine was added to obtain

FIG. 3. Another example of change of membrane current during depolarization after addition of acetylcholine to Tyrode solution to a concentration of 10^{-4} g/ml. FIG. A is the same as in FIG. 2A. The membrane current is shown in FIG. B as follows. B1: in the control state; B2: 1.5 minutes after addition of acetylcholine to a concentration of 10^{-4} g/ml; B3: after 2.5 minutes; B4: after 5 minutes.

a final concentration of 10^{-5} g/ml. Immediately after its addition there was a decrease of positive outward current. It recovered and increased gradually, exceeding the control value in about three minutes. In this experiment this increase became more marked when acetylcholine was added further to obtain a final concentration of 10^{-3} g/ml. The same initial decrease and later increase of the positive outward current are noticed in FIG. 3, in which the initial inward current is seen. Acetylcholine was added to a concentration of 10^{-4} g/ml.

Effects of acetylcholine were examined when the NaCl of Tyrode solution was replaced by sucrose. It was found that the increase of the positive outward current due to acetylcholine was more marked than in the Tyrode

FIG. 4. Change of membrane current during depolarization after addition of acetylcholine to sucrose-Tyrode solution to a concentration of 10^{-4} g/ml. FIG. A is the same as in FIG. 2A. The membrane current is shown in FIG. B as follows. B1: in the control state; B2: 1.5 minutes after addition of acetylcholine to a concentration of 10^{-4} g/ml; B3: after 3 minutes; B4: after 5 minutes.

solution (FIG. 4). This indicates that the increase of potassium current was induced by acetylcholine.

The g_{Na} and the g_K were calculated at each voltage and time as shown in FIG. 5. In this calculation the sodium current change ΔI_{Na} was obtained by subtracting the potassium current change ΔI_K from the total current change ΔI_m . This ΔI_m was obtained as the difference between the current, when acetylcholine was added (to 10^{-4} g/ml in FIG. 5) to the normal Tyrode solution, and that before its addition. The ΔI_K was obtained as the difference between the current, when acetylcholine was added to the Na-free solution which was obtained by replacing the NaCl of Tyrode solution by sucrose, and that before its addition. A change of the g_{Na} was noticed in addition

FIG. 5. Changes of the chord conductance in depolarization by addition of acetylcholine to a concentration of 10^{-4} g/ml. Abscissa shows the time after the onset of depolarization. Ordinate shows the chord conductance. Resting and clamp potentials of the preparation are indicated on the potential schema.

to the g_K change, as FOZZARD and SLEATOR¹¹⁾ described with acetylcholine effect on the ionic current of the atrial muscle fibers, although acetylcholine concentration was much higher in this experiment. Compared with the control state, the g_K decreased soon after the addition of acetylcholine throughout the whole duration of depolarization, but increased later. The g_{Na} increased in the first phase of depolarization up to about 150 msec, but beyond this phase it decreased progressively. Namely, sodium inactivation became more rapid than in the control state.

DISCUSSION

Our result on the normal control state showed some difference from that of a similar experiment by DECK and TRAUTWEIN^{5,6)}. The decrease of g_{Na} was more rapid in our result than in their result, although it must be admitted that the inactivation of g_{Na} was slow and incomplete, as they described. The g_K decreased markedly immediately after depolarization and increased rapidly later up to about 100 msec and slowly thereafter in our result, while the g_K continued to decrease after depolarization in their result. Since the delayed increase of the g_K was slight, our result cannot be very definite, but it is rather closer to the results by HALL, HUTTER and NOBLE¹⁴⁾ and MCALLISTER and NOBLE²³⁾.

According to our result the repolarization phases of the action potential of Purkinje fibers can be explained as follows: there is a time-dependent change of the g_K early in the phase, especially up to 100 msec. As shown by the conductance at -10 mV and -16 mV in FIG. 1, the g_{Na} and g_K show a voltage-dependent change, inducing a change in the time at which the g_{Na} and g_K cross each other. The plateau of the action potential is formed because the decrease of g_{Na} is relatively smaller and the increase of g_K is initially not marked. But the g_{Na} shows a time-dependent decrease, becoming especially small beyond 100 msec of depolarization, and the g_K shows a gradual increase, which causes an outflow of potassium ions from the cell and a decrease of the membrane potential. This induces a marked decrease of g_{Na} and a less marked increase of g_K . Eventually the g_{Na} becomes smaller than g_K , and phase 3 of the action potential is produced.

The above-mentioned g_{Na} and g_K were obtained on the conventional assumption that the ionic current of the cell membrane consists chiefly of Na^+ and K^+ and the potassium current can be measured when the NaCl of Tyrode solution was replaced by sucrose, glucose or choline chloride, but recently several authors have more or less cast doubt on the application of this assumption to Purkinje fibers. GLITSCH and HAAS¹³⁾ observed that the K flux evidently changed when extracellular Na was replaced by choline. DECK and TRAUTWEIN⁶⁾ showed that, in the case of Purkinje fibers, replacement of

sodium by choline or glucose virtually abolishes the outward current which occurs immediately following depolarizing clamps. Employing K^{42} efflux measurements under voltage clamp conditions, HAAS and KERN¹³⁾ also found a larger value of the K net flux in the range of depolarization than that determined by electrical methods, although they noticed that both methods in general yielded qualitatively similar results.

Further studies by several authors have revealed that ionic currents for chloride and calcium and other unknown negative non-sodium currents cannot be ignored in the electrical activity of Purkinje fibers. But there are still many unknown details and serious disagreements. FOZZARD and SLEATOR¹¹⁾ estimated the chloride conductance to be, on an average, 12% of the g_K of the resting membrane. DUDEL, PEPER, RÜDEL and TRAUTWEIN⁸⁾ found that the chloride current in relation to the relative contribution to the net current increased from 20% at rest to 50% or more at positive potentials in the steady state and that its peak was delayed by about 20 msec from the start of the depolarization, being inactivated with a time constant of 50 msec. REUTER^{26,27)} found that a slow inward current was carried by calcium ions, but DUDEL, PEPER and TRAUTWEIN⁹⁾ found no appreciable calcium current to flow in the range of membrane potentials covered by the action potential. DUDEL, PEPER, RÜDEL and TRAUTWEIN^{7,31)} proposed the presence of a "negative non-sodium" current other than the calcium current, the carrier of which was unknown.

With this information as reference, our results mentioned above should be reconsidered. As for the problem described at the beginning of the DISCUSSION, further reports have appeared after DECK and TRAUTWEIN^{5,6)}. While DECK and TRAUTWEIN⁵⁾ and MCALLISTER and NOBLE²³⁾ proposed a slow or an incomplete inactivation of g_{Na} , and REUTER²⁷⁾ also endorsed it, DUDEL, PEPER, RÜDEL and TRAUTWEIN⁷⁾ found a rapid and almost complete inactivation of g_{Na} and no component of delayed rectification of g_K in contrast to VASALLE³²⁾ and MCALLISTER and NOBLE^{23,24)}. According to our results, even in the experiments, in which the g_K was obtained by replacement of NaCl of the normal Tyrode solution by choline-chloride, the g_K showed a delayed increase after depolarization. In this case the chloride current component may be included in the g_K at the beginning of the depolarization. By subtracting this component the result would show more markedly that the real potassium conductance increases with delay. Since MCALLISTER and NOBLE²³⁾ and others^{5,13)} showed that the delayed increase in g_K after depolarization is depressed in the sodium free solution, the real potassium conductance would be larger than our g_K .

Further accurate revision of the above-mentioned g_{Na} and g_K seems to be impossible in view of the lack of agreement at present. For instance, our finding of an acetylcholine effect described as an increase of the g_{Na} especially in the early phase of depolarization might be related to an increase of calcium

current instead, but no reliable data seem to be present to permit a revision on the ionic currents other than sodium and potassium under the influence of acetylcholine or any other drugs. For a while they must be regarded as an inward and outward conductance obtained by the method described.

Most studies admitted that acetylcholine has little effect on the electrical activity of the cardiac ventricle. But earlier studies of LUEKEN and SCHÜTZ²²⁾ with suction electrodes showed that 1:5,000 acetylcholine did speed repolarization in the frog ventricle and HOFFMAN¹⁹⁾ saw a similar effect in records of transmembrane potential from single fibers of the frog ventricle. As for the mammalian ventricle HOFFMAN and SUCKLING¹⁸⁾ noticed, with the papillary muscles of the dog ventricle, no change of the action potential and the resting potential in a concentration up to $5.5 \times 10^{-4}M$ and a very slight increase in the slope of phase 2 and a corresponding decrease in the slope of phase 3 in a very high concentration ($11 \times 10^{-2}M$). SCHMIDT²⁸⁾ showed that a high concentration of $10^{-4}g/ml$ of acetylcholine shorten the action potential of Purkinje fibers by about 10%. SUZUKI, SATO, IDA, HIRAOKA and SANO³⁰⁾ noticed in the rabbit papillary muscle fibers that acetylcholine in a concentration of $10^{-3}g/ml$ shortened the duration of the action potential first with an increase in the slope of phase 3 and prolonged it later with the prolongation of phase 3. They³⁰⁾ also observed that in the dog Purkinje fiber acetylcholine in $10^{-4}g/ml$ prolonged slightly the duration of the action potential with the prolongation of phase 3.

In spite of the scantiness of the reports in which were noticed changes of electrical activity of the Purkinje fibers or ventricular muscle fibers, the number of reports which described either the positive^{2,3,10,17,21,25)}, negative or biphasic^{10,20)} inotropic effect of acetylcholine on the ventricle is increasing, although much controversy still exists. For instance, BUCCINO, SONNENBLICK, COOPER and BRAUNWALD²⁾ described its positive inotropic effect in a concentration of 10^{-5} to $3 \times 10^{-4}g/ml$ and frequently a small negative effect in a concentration of 10^{-9} to $10^{-7}g/ml$ on the cat papillary muscle. SCHMIDT²⁸⁾ found an initial negative inotropic action followed by a positive inotropic action by 10^{-5} to $10^{-4}g/ml$ of acetylcholine without noticing any change of resting potential and the action potential. SUZUKI, SATO, IDA, HIRAOKA and SANO³⁰⁾ noticed a similar biphasic inotropic action, but found some correlation with the above-mentioned changes of the action potential in a higher concentration. In this study, although a biphasic inotropic action was noticed up to $10^{-5}g/ml$, no distinct change of the action potential could be noticed at this lower concentration.

In the present study, no significant change in the ionic currents of Purkinje fibers was noticed up to the final concentration of $10^{-6}g/ml$ of acetylcholine, but some changes were noticed, more or less frequently, in a concentration higher than $10^{-5}g/ml$. Our result showing an initial decrease of

current and a later increase by acetylcholine with Purkinje fibers seems to have some relation with that of SUZUKI, SATO, IIDA, HIRAOKA and SANO⁸⁰⁾, which showed that the isotonic contraction of isolated rabbit papillary muscles showed a slight decrease soon after the addition of acetylcholine and an increase thereafter. Although the electrical phenomenon of Purkinje fibers should not be directly correlated with the mechanical action of ventricular muscle fibers, the lack of changes of the action potential or resting potential of Purkinje fibers and ventricular muscle fibers in a concentration of about 10^{-5} g/ml may be merely an outward appearance. Otherwise the relatively larger ionic currents might be due to a slightly depolarized resting state occasionally found in this technic.

At this time when there are marked differences of opinions on the calcium current, it seems to be too much speculation to consider the relation between these obtained values of assumed g_{Na} and g_K with such controversial findings of inotropic action of acetylcholine on the ventricle, especially because our findings were obtained with Purkinje fibers and not with the ventricular muscle fibers.

SUMMARY

Ionic currents in excitation and effects of acetylcholine on them were examined with isolated young calf Purkinje fibers by means of the voltage clamp technique. It was found that in the control state the g_{Na} , which increased markedly at the beginning of depolarization, decreased rapidly until about 50 msec and thereafter slowly. The g_K , which showed a marked decrease at the beginning of depolarization, showed a fairly rapid increase until 100 msec and a gradual increase thereafter.

Acetylcholine showed no significant effects on the ionic current up to the concentration of 10^{-6} g/ml. In a concentration higher than 10^{-5} g/ml the g_K decreased soon after the addition of acetylcholine, but increased later. The g_{Na} increased during the first phase of depolarization up to about 150 msec, but beyond this phase it decreased progressively.

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