

## Control of Action Potential Duration by Calcium Ions in Cardiac Purkinje Fibers

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**ABSTRACT** It is well known that cardiac action potentials are shortened by increasing the external calcium concentration ( $Ca_o$ ). The shortening is puzzling since Ca ions are thought to carry inward current during the plateau. We therefore studied the effects of  $Ca_o$  on action potentials and membrane currents in short Purkinje fiber preparations. Two factors favor the earlier repolarization. First, calcium-rich solutions generally raise the plateau voltage; in turn, the higher plateau level accelerates time- and voltage-dependent current changes which trigger repolarization. Increases in plateau height imposed by depolarizing current consistently produced shortening of the action potential. The second factor in the action of Ca ions involves  $i_{K_1}$ , the background K current (inward rectifier). Raising  $Ca_o$  enhances  $i_{K_1}$  and thus favors faster repolarization. The Ca-sensitive current change was identified as an increase in  $i_{K_1}$  by virtue of its dependence on membrane potential and  $K_o$ . A possible third factor was considered and ruled out: unlike epinephrine, calcium-rich solutions do not enhance slow outward plateau current,  $i_x$ . These results are surprising in showing that calcium ions and epinephrine act quite differently on repolarizing currents, even though they share similar effects on the height and duration of the action potential.

### INTRODUCTION

The duration of the cardiac action potential is sensitive to the external calcium concentration. As a general rule, elevated concentrations shorten the action potentials and reduced concentrations lengthen it. Such behavior has been observed in the electrocardiogram and in Purkinje fibers (Dudel et al., 1966; Temte and Davis, 1967; Weidmann, 1964) as well as ventricular muscle (Hoffman and Suckling, 1956; Dudel and Trautwein, 1958). Although these effects have been known for some time, their explanation remains unclear.

Another effect of external calcium is to raise the action potential plateau. The factors controlling the height of the plateau are somewhat better understood. Here it is generally agreed that  $Ca^{++}$  ions play an important role as charge carriers (for reviews, see Reuter, 1973; Trautwein, 1973; Cranefield, 1975). Calcium contributes to the slow inward current which dominates the first 100–200 ms of the action potential and which helps support the plateau. Increasing  $Ca_o$  enhances the slow inward current and thereby elevates the plateau (see, for example, Reuter, 1967).

The existence of a pathway for  $Ca^{++}$  influx does not directly explain why the

action potential is shortened by elevated  $Ca_o$ . If anything, one might expect that calcium-rich solutions should delay the repolarization by favoring inward current over the plateau range of potentials. To explain the generally observed behavior, it seems necessary to suppose that calcium ions have an additional influence, possibly on a membrane current other than the slow inward current. This type of mechanism has already been demonstrated in the case of epinephrine and other  $\beta$ -adrenergic compounds. Epinephrine resembles calcium ions in abbreviating the action potential, even at constant driving rates. This effect takes place despite a calcium-like elevation of plateau level (Reuter, 1967; Carmeliet and Vereecke, 1969). The shortening of the action potential is mediated by an increase in slow outward plateau current,  $i_x$  (Tsien et al., 1972), which is sufficient to overcome the enhanced slow inward current. The results with epinephrine raised the possibility that calcium ions might also increase  $i_x$  (cf. Beeler and Reuter, 1970; Kass and Tsien, 1975). This question was the starting point of the present study. Our voltage clamp experiments showed that epinephrine and calcium act differently on repolarization currents, even though their effects on the action potential are remarkably similar.

#### METHODS

Purkinje fiber bundles were obtained from either ventricle of young calf hearts. Action potentials and membrane currents were recorded in short (1–2 mm) preparations using the two-microelectrode voltage clamp method of Deck et al. (1964) with some modifications (see Tsien, 1974 and Kass and Tsien, 1975 for details).

Action potential or membrane current records show the effects of external calcium concentration (abbreviated  $Ca_o$ ) in the steady state. In any given experiment, action potentials were driven by external stimulus pulses, applied at a constant rate (usually 0.5 or 0.33 Hz). During voltage clamp runs, clamp pulses were also applied at the same rate. The effects of calcium on the action potential were monitored in each of the preparations used to illustrate changes in membrane current. Some preparations became partially depolarized due to imperfect sealing at the impalement sites or at the cut ends. In these cases, action potentials were evoked while passing a steady hyperpolarizing current (cf. Tsien, 1974, Fig. 3). Such action potentials showed a normal plateau, indicating that repolarization mechanisms were basically unchanged. They responded to variations in  $Ca_o$  in a manner similar to Fig. 1 A. Action potential duration was defined as the period between the upstroke and repolarization to  $-60$  mV.

#### RESULTS

##### *Comparison between Effects of Epinephrine and Calcium Ions*

Fig. 1 compares the influence of calcium ions and epinephrine on the action potential. The traces are oscilloscope records from consecutive runs during a continuous microelectrode impalement. In the first run (panel A)  $Ca_o$  was changed from 7.2 to 1.8 mM. The illustrated effect of  $Ca_o$  was typical of other experiments over the range between 0.9 and 7.2 mM. Calcium-rich solution elevated the plateau (Dudel et al., 1966; Reuter, 1967) while also shortening the action potential (Dudel et al., 1966; Temte and Davis, 1967; Weidmann, 1964). The two effects were fully reversible; they occurred simultaneously during the

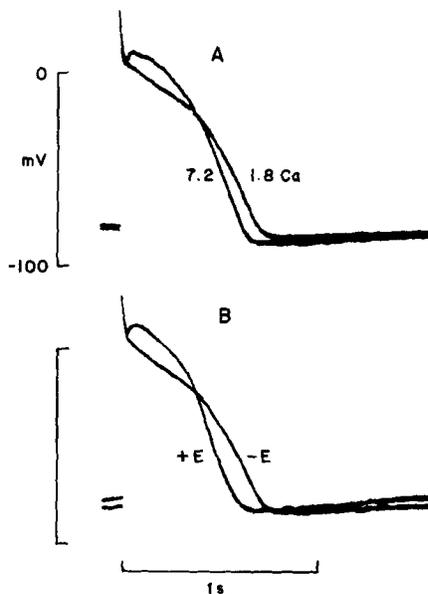


FIGURE 1. Effects of Ca and epinephrine on Purkinje fiber action potentials. Storage oscilloscope records showing steady-state effects. (A) Action potentials driven at 30/min in 7.2 mM  $Ca_o$ , and 17 min after reducing  $Ca_o$  to 1.8 mM. (B) Action potentials in 1.8 mM  $Ca_o$ , before and 11 min after exposure to  $10^{-7}$  M epinephrine. Drive rate was increased to 40/min to avoid possible spontaneous activity in epinephrine. Preparation 123-1.

change of solution, and reached a steady state within 5 min or so.

Panel B illustrates the effect of epinephrine ( $10^{-7}$  M) in the same preparation, with  $Ca_o$  kept constant at 1.8 mM. It is evident that the influence of the sympathetic hormone is quite comparable to the effect of elevated calcium. This observation naturally raises the question of whether the effects on membrane currents also are similar.

Fig. 2 shows an experiment which addresses this question. Plateau currents (lower traces) were recorded during the application of a standard voltage clamp pulse (upper traces). The pulse duration was set at 400 ms to allow comparison with changes in the action potential, and the holding potential was set at  $-25$  mV to inactivate the transient outward chloride current (Dudel et al., 1967 *a*; Fozzard and Hiraoka, 1973) as well as the excitatory sodium current (Weidmann, 1955 *a*). The sequence of solutions followed the same pattern as in Fig. 1, proceeding from calcium rich solution (trace *a*) to normal  $Ca_o$  (trace *b*), and then to normal  $Ca_o$  Tyrode +  $10^{-7}$  M epinephrine (trace *c*). Finally, calcium-rich solution without epinephrine was reintroduced to demonstrate reversibility (trace *d*).

The superimposed records allow direct comparison of the actions of calcium (left) and epinephrine (middle). It is immediately evident that these agents act differently on membrane currents. The traces in the middle panel cross each other, while those on the left remain widely separated during the clamp pulse.

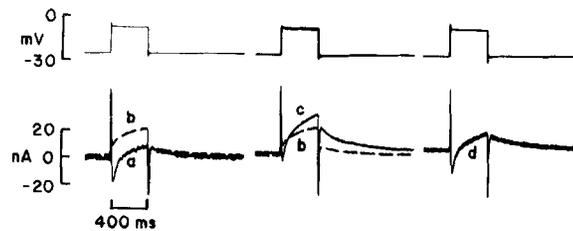


FIGURE 2. Effects of Ca and epinephrine on membrane currents over the plateau range. Superimposed chart recordings of voltage clamp pulses from  $-25$  to  $-8$  mV (above) and accompanying current traces (below). The sequence of solution changes was (a)  $5.4$  mM  $\text{Ca}_o$ , (b)  $1.8$  mM  $\text{Ca}_o$ , after 27 min, (c)  $1.8$  mM  $\text{Ca}_o + 10^{-7}$  M epinephrine after 9 min, (d)  $5.4$  mM  $\text{Ca}_o$  without epinephrine, after 29 min. "Spikes" on make and break of voltage pulse lasted less than 3 ms and reflect slight ringing in the clamp. Preparation 162-1, apparent cylindrical area  $0.005$  cm $^2$ . See Reuter (1974, Fig. 1) for comparison with ventricular muscle.

In interpreting these results, it is helpful to distinguish between changes in membrane current during the first 50 ms of clamp depolarization and those later on. Epinephrine and calcium ions influence the early current in a qualitatively similar way, giving it a marked inward peak. This is due to an enhancement of the slow inward current, as previously reported in Purkinje fibers (Reuter, 1967; Vitek and Trautwein, 1971). The effects first become different later on during the pulse, as the slow inward current inactivates and as the net current becomes outward. Epinephrine strongly promotes delayed rectification ( $i_x$ ), to the point of causing  $c$  to cross  $b$  as noted above. An increase in delayed rectification is also indicated by the very much larger tail of outward current after the clamp pulse. The combination of augmented late current and larger tail current is accounted for by an enhancement of the slow outward plateau current,  $i_x$  (Noble and Tsien, 1969). A previous report has already illustrated increases in  $i_x$  produced by monobutyl cyclic adenosine monophosphate (cAMP) (Tsien et al., 1972).

Returning to the left panel of Fig. 2, it is apparent that increasing  $\text{Ca}_o$  does not promote delayed rectification, as we had originally suspected. This point is demonstrated most clearly by the tails after the pulse. In calcium-rich solution the tail amplitude is, if anything, slightly smaller. Thus, the conductance of the  $i_x$  channels was not increased (see also Kass and Tsien, 1975). The enhancement of  $i_x$  by epinephrine provides a straightforward explanation for how the sympathetic hormone shortens the action potential (Tsien et al., 1972). The present results indicate that some other mechanism must mediate the effect of elevated calcium concentration. The rest of this paper will be concerned with determining this mechanism.

#### *Effect of Calcium on All-or-Nothing Repolarization*

In Fig. 2 and in other experiments, calcium-rich solution displaced the current trace in the inward direction over the entire duration of the depolarizing clamp pulse. One interpretation is that the higher  $\text{Ca}_o$  promotes steady current through the slow inward current pathway due to its incomplete inactivation (Reuter, 1968; Trautwein et al., 1975; Gibbons and Fozzard, 1975; Kass and

Tsien, 1975). Whatever the explanation, the voltage clamp results are puzzling in relation to the effect of  $Ca_o$  on action potential duration. The repolarization process depends upon the development of net outward current over the voltage range between the plateau and the resting potential. One would expect that, by favoring inward current, elevated  $Ca_o$  would cause a delayed terminal repolarization, the opposite of what actually happens.

To resolve this difficulty, we tried to get more information on the repolarization process while interfering as little as possible with the action potential. One approach was to study the effect of calcium ions on all-or-nothing repolarization. This term refers to the regenerative repolarization which can be evoked during the action potential by hyperpolarizing beyond a critical threshold (Weidmann, 1951). Changes in this threshold level reflect underlying time-dependent changes in the membrane current-voltage relationship (see Noble and Tsien, 1972; Goldman and Morad, 1976<sup>1</sup>). As the net  $I/V$  relation changes progressively toward outward current, the threshold level moves in the positive direction. Disappearance of the regenerative repolarization threshold occurs when the  $I/V$  relation becomes sufficiently outward to lack a region of inward current positive to the resting potential. The question here is whether Ca ions hasten the disappearance of this threshold.

Fig. 3 illustrates the influence of  $Ca_o$  on the threshold for regenerative repolarization, using the procedure of Vassalle (1966). Panel A shows the action potential in 1.8 mM  $Ca_o$ , together with responses to hyperpolarizations just beyond and just short of threshold (dashed traces). Triangles indicate other threshold determinations. As expected, the threshold level becomes progressively less negative with time (Vassalle, 1966). After the last threshold determination, at 550 ms (arrow), no regenerative repolarization could be evoked. Panel B shows the effect of raising  $Ca_o$  to 7.2 mM. The action potential duration decreased from 1,070 to 880 ms, and this abbreviation was accompanied by a shorter period for all-or-nothing repolarization, 370 ms (arrow). Thus, most of the abbreviation took place during the first part of the action potential, when time-dependent current changes were rate limiting. In comparing A and B, it is evident that the repolarization threshold lies at about the same level early in the plateau, but moves more rapidly in the depolarizing direction in the calcium-rich solution.

The conclusion from these results is that the net membrane  $I/V$  relation becomes outward faster in the presence of high  $Ca_o$ . This acceleration is consistent with the decreased action potential duration, but does it conflict with the direct recordings of membrane current in Fig. 2? One difference between these experiments lies in the membrane potential itself. The level of the clamp pulse was deliberately fixed in the voltage clamp experiments, but the level of the plateau changed with  $Ca_o$  in Fig. 3. The major plateau currents (slow inward current and  $i_x$ ) are quite sensitive to membrane potential. Thus, the higher plateau in calcium-rich solution could be important in favoring changes in these currents that in turn lead to faster repolarization.

<sup>1</sup> Goldman, Y., and M. Morad. The apparent threshold for repolarization of the frog ventricular action potential: a time and potential dependent phenomenon. Manuscript submitted for publication.

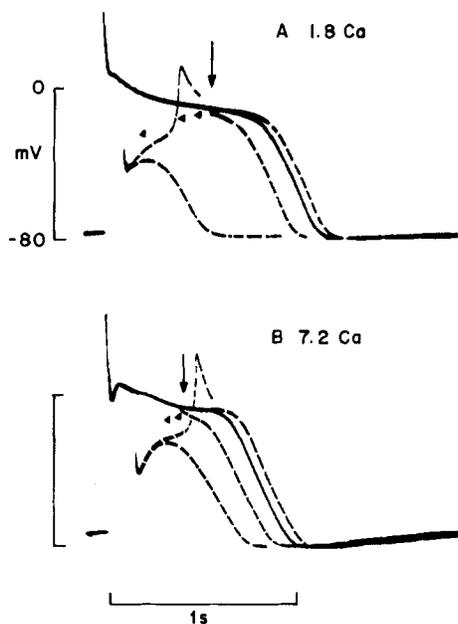


FIGURE 3. Influence of Ca on the threshold for all-or-nothing repolarization. Solid traces show action potentials driven at 30/min in 1.8 mM  $Ca_0$  (A) and after elevating  $Ca_0$  to 7.2 mM (B). Dashed traces are responses to 20-ms voltage clamps to potentials just beyond and just short of the repolarization "threshold" (defined as in McAllister et al., 1975). Arrows mark the latest occurrence of regenerative repolarization that could be obtained. No threshold could be determined 30–50 ms later. Triangles mark other threshold determinations (traces omitted for the sake of clarity).

#### *Effect of Plateau Level on Action Potential Duration*

As a simple test of this hypothesis, we altered the plateau level by means of intracellularly applied currents, and looked for effects on the subsequent repolarization. Fig. 4, panel A shows the basic result. A depolarizing current pulse (lower trace) was applied to a short Purkinje fiber preparation 50 ms after the beginning of a stimulated action potential. The plateau level was raised by about 5 mV above the control plateau. After the pulse, however, the situation is quickly reversed; repolarization is faster, and the duration of the modified action potential is about 125 ms briefer than the control action potential.

Panel B shows superimposed records from another preparation where the magnitude of the current pulse was varied (see legend for details). Increasing the strength of the depolarizing pulse raised the plateau and abbreviated the action potential in a graded manner. Conversely, a hyperpolarizing pulse produced a longer action potential. Current pulse experiments like these were carried out in a total of eight short preparations. In each case, elevation of the plateau was accompanied by a decrease in action potential duration. It is interesting that similar experiments in ventricular muscle have produced rather different results (see Discussion).

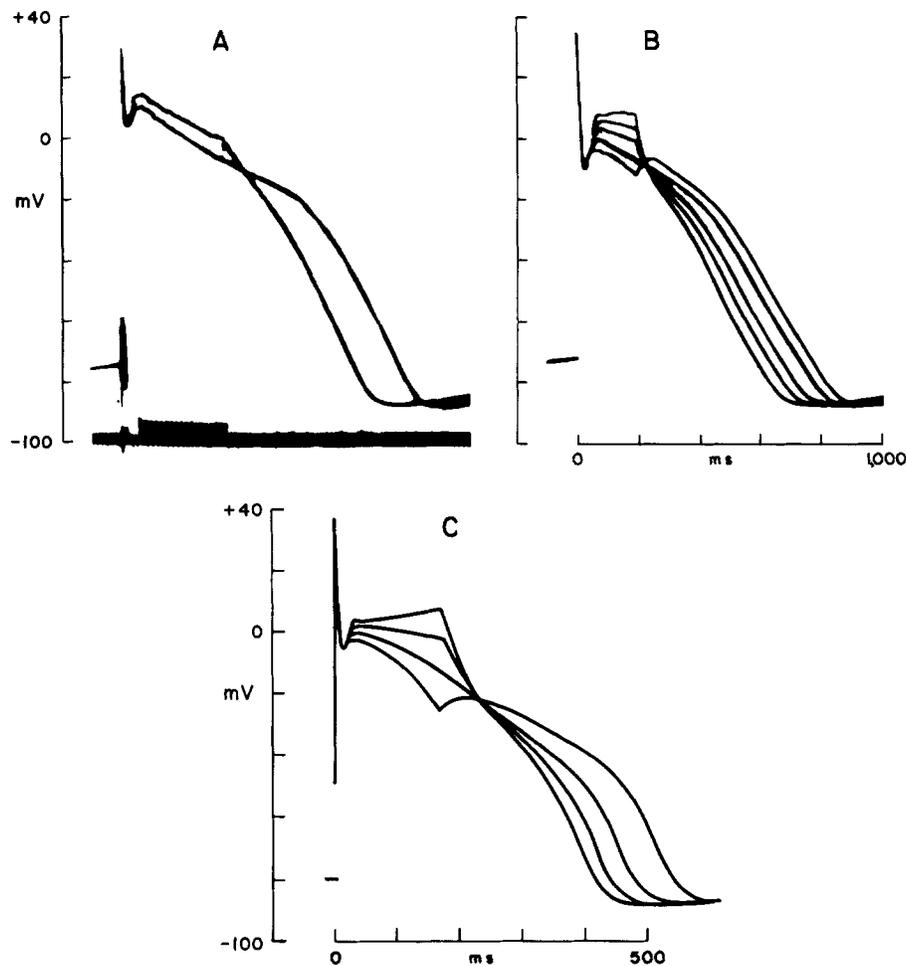


FIGURE 4. Imposed changes in plateau level and their effect on action potential duration. Unmodified action potentials are superimposed on action potentials where the plateau was altered by applied current. A and B show oscilloscope records of experimental results. (A)  $2.27 \mu\text{A}/\text{cm}^2$  depolarizing current pulse (lower trace), preparation 132-2, time scale as in B. (B) Graded effect of changes in plateau level produced by current pulses (not shown) of magnitude 11.4, 6.3, 3.4, and  $-3.7 \mu\text{A}/\text{cm}^2$ , preparation 134-2. (C) Theoretical results using the action potential model of McAllister et al., 1975, Fig. 4. Calculated effects of current pulses of magnitude 10, 5, and  $-5 \mu\text{A}/\text{cm}^2$ . Computed plateau currents were compared at the crossover point ( $-23 \text{ mV}$ ) between the control trace and the most strongly modified action potential. The elevated plateau increased  $i_{\text{r}}$  from 2.32 to  $2.88 \mu\text{A}/\text{cm}^2$  and decreased the slow inward current ( $i_{\text{si}}$ ) from  $-2.09$  to  $-1.51 \mu\text{A}/\text{cm}^2$ .

The responses to brief potential displacements run counter to the behavior expected for a passive RC circuit. They might be accounted for, however, by the participation of conductance changes which are voltage dependent as well as time dependent. In pursuing this explanation, we investigated the effects of current pulses on a theoretical Purkinje fiber action potential (McAllister et al.,

1975) based on voltage clamp results. In the model, repolarization is controlled by two components, the slow inward current, which turns off, and the slow outward K current,  $i_x$ , which turns on. Fig. 4 C shows that the effect of applied current during the plateau can be reproduced by the computer model. There is a reasonable similarity between the calculated repolarizations and the experimental records in panel B. The theoretical results show a decrease in action potential duration which may be accounted for by a dual effect of membrane potential. Raising the plateau promotes a greater degree of  $i_x$  activation while also hastening the inactivation of the slow inward current (see Fig. 4 legend for details). According to the model, both of these factors are important. But revision of this account may be necessary when we gain more information on the inactivation of the slow inward current in Purkinje fibers (see Discussion).

#### *Another Action of Ca?*

The preceding results indicated that increases in plateau level per se can lead to shortening of the action potential. Thus, it seemed possible that all the effects of calcium-rich solutions involve an elevated plateau arising from augmented calcium influx. But in studying the effects of current pulses, we came across evidence that some additional mechanism must also participate.

Fig. 5 shows an experiment in a preparation with a rather high and square plateau. The left and right panels illustrate the effects of  $Ca_o$ , which were different from those shown elsewhere in this paper (Figs. 1 A and 6). Reduction of  $Ca_o$  from 7.2 to 1.8 mM (left) prolonged the action potential by about 100 ms with a barely noticeable lowering of the plateau. Restoring  $Ca_o$  from 1.8 to 7.2 mM (right) decreased action potential duration by 150 ms without any detectable change in plateau level. These results may be compared with the effect of a depolarizing current pulse (middle) in 1.8 mM  $Ca_o$ . Raising the plateau does shorten the action potential, as in Fig. 4. However, the amount of shortening is

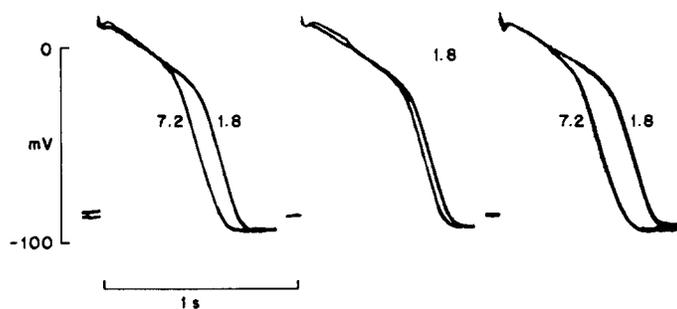


FIGURE 5. Action potentials in a Purkinje fiber preparation with an unusually high and "square" plateau. Steady-state effects of Ca (left and right panels) are compared with effect of plateau level (middle panel). (Left) Action potentials in 7.2 mM  $Ca_o$  and after lowering  $Ca_o$  to 1.8 mM. (Middle) Effect of  $1.1 \mu A/cm^2$  depolarizing current pulse during the plateau in 1.8 mM  $Ca_o$ . The modified action potential repolarizes earlier than the control. (Right) Action potentials in 1.8 mM  $Ca_o$  and after restoring  $Ca_o$  to 7.2 mM. Preparation 130-1.

small compared to the effects of  $Ca_o$ , even though the current pulse produced a greater plateau elevation.

Apparently, calcium-dependent shortening of the action potential can sometimes be dissociated from elevation of the plateau. In this sense, the present example is consistent with earlier observations of Temte and Davis (1967) and Dudel et al. (1966). These findings do not rule out the idea that plateau level helps control action potential duration in other preparations where the plateau is elevated by calcium. The preparation in Fig. 5 is atypical of our experiments in calf Purkinje fibers. We generally find much lower and less square plateaus in 1.8 mM  $Ca_o$ , and clear increases in the height of the plateau in calcium-rich solutions, as previously reported by Reuter (1967) and Carmeliet and Vereecke (1969).

#### *Influence of Calcium Ions on Time-Independent Current*

In searching for an additional mechanism for calcium action, we considered the possibility that calcium ions might influence a time-independent membrane current. In an earlier study, Dudel et al. (1966) found that increasing  $Ca_o$  from 2.7 to 21.6 mM shifted the current-voltage relationship in a manner favoring repolarization. Their results were compatible with an abbreviation of the action potential without an increase in plateau height, as found in the case of Fig. 5 or their Fig. 9 A. The change in net membrane current was tentatively attributed to a decrease in sodium conductance. We have reexamined this point, and have also found alterations in the current-voltage relationship with  $Ca_o$ . However, the characteristics of the calcium effect are different than those previously reported, and they suggest a different ionic basis. Our evidence indicates that calcium-rich solutions increase the potassium current  $i_{K1}$ .

Voltage clamp experiments were designed to obtain information on membrane characteristics under conditions closely resembling the action potential repolarization. The basic procedure is illustrated in the upper panel of Fig. 6. The membrane was clamped at  $-50$  mV to inactivate the excitatory sodium current. From this holding potential, a double pulse waveform was applied once every 3 s. The first pulse depolarized the membrane to  $-2$  mV for 400 ms, and activated the various plateau currents. The second pulse repolarized the potential to a variable level  $V$ , so that membrane current could be measured over the range of potentials where action potential repolarization occurs. In the example in Fig. 6,  $V$  is  $-59$  mV. Superimposed on the clamp waveform are records of the action potential, obtained in consecutive runs in 7.2, 0.9, and 7.2 mM  $Ca_o$ . The action potentials were stimulated at the same steady rate of 0.33 Hz. It is apparent from the records that the influence of  $Ca_o$  is similar to the effects in Fig. 1 A, and that the first clamp pulse roughly coincides with the action potentials in level and duration.

The middle panels illustrate membrane currents accompanying the double pulse waveform shown above. As in Fig. 2, the early inward peak is enhanced by the calcium-rich solution. There is an additional effect in this case: an increase in a transient outward current (arrow) preceding the inward peak. The outward transient is the chloride current described by Dudel et al. (1967 *a*) and Fozzard

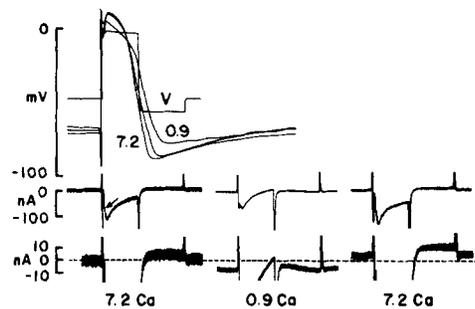


FIGURE 6. Effect of Ca on membrane current over the voltage range where terminal repolarization occurs. (Upper panel) Imposed voltage clamp waveform superimposed on action potentials which were stimulated during application of roughly 8-nA hyperpolarizing current. Action potentials were recorded in consecutive runs in 7.2, 0.9, and 7.2 mM  $Ca_o$ . The briefest action potential was obtained after returning to 7.2  $Ca_o$ . (Middle and lower panels) Currents accompanying the clamp waveform shown above, taken during the same three runs at low gain (middle) and five times higher gain (bottom). All traces are chart recordings; full action potential overshoot not registered. Arrow indicates transient chloride current. Preparation 160-2, apparent cylindrical area 0.011  $cm^2$ .

and Hiraoka (1973). Its enhancement here accounts for the deep notch in the action potential in high  $Ca_o$  (upper panel). A corresponding effect was not observed in Fig. 2, but this may have been due to the less negative holding potential and its effect on chloride current inactivation (Fozzard and Hiraoka, 1973).

The main feature of interest in Fig. 6 is the effect of  $Ca_o$  on time-independent currents near  $-50$  mV. The lower records show the current signals recorded at very high amplification. The current traces at  $-59$  and  $-50$  mV show little time dependence, and are clearly more outward in the calcium-rich solution (compare left and right panels with middle panel). These changes are small relative to the effects at  $-2$  mV (see middle traces) but they would be very important nevertheless in modifying the rate of repolarization.

The next question is the ionic basis of the calcium effect. Here the high gain records provide an important clue. In the presence of 7.2 mM  $Ca_o$  (left and right panels) the current is more outward at  $-59$  mV than at  $-50$  mV. This is not the case in 0.9 mM  $Ca_o$ . Apparently, calcium-rich solution can give the current-voltage relationship a negative slope over this range of potentials. The negative slope characteristic is interesting because it is a distinctive property of inwardly rectifying potassium currents in Purkinje fibers (Trautwein et al., 1965; McAllister and Noble, 1966; Dudel et al., 1967 *b*; Noble and Tsien, 1968). Conversely, one might not expect very much negative slope to arise from a decrease in inward leak current carried by sodium, since the current-voltage relationship of such a component would not be steeply voltage dependent.

#### *Evidence that Calcium Increases $i_K$ , the Inward Rectifier*

Further experiments were carried out to see if the calcium-dependent current change had other characteristics expected for a potassium conductance. We

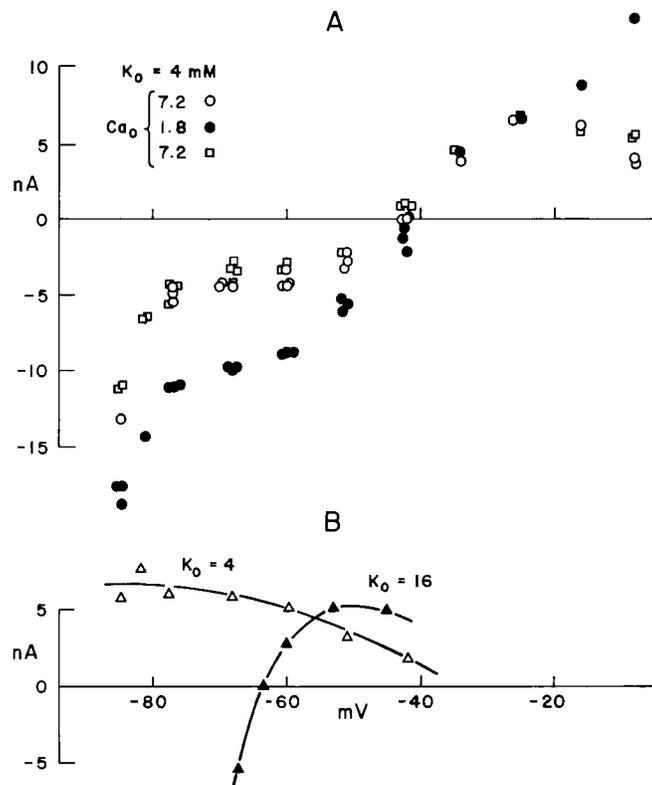


FIGURE 7. Ca-dependent changes in membrane  $I/V$  relationships. (A) Current-voltage relationships measured in 7.2 and 1.8 mM  $Ca_o$  with  $K_o$  kept constant at 4 mM. Net membrane current ( $I$ ) is plotted against pulse potential ( $V$ , see Fig. 6). For most values of  $V$  the current trace was flat after a brief inward transient. When slow current changes did occur,  $I$  was taken as the peak outward or minimum inward current during the pulse. (B)  $\Delta I/V$  relations. The difference between currents measured in 7.2 and 1.8 mM  $Ca_o$  ( $\Delta I$ ) is plotted for 4 mM  $K_o$  (from data in A) and for 16 mM  $K_o$  (data not shown). Smooth curves drawn by eye. The  $\Delta I/V$  relationships are not extended positive to  $-40$  mV where steady slow inward current may become important. Same preparation as in Fig. 2.

studied the effect of  $Ca_o$  over a wide range of potentials and at two different potassium ion concentrations (Fig. 7). Panel A shows the current-voltage relationships in 4 mM  $K_o$ , determined by means of the protocol in Fig. 6. The data were obtained from records in three consecutive runs, in 7.2, 1.8, and 7.2 mM  $Ca_o$ . The plot shows that calcium-rich solution shifted the current-voltage relationship in the outward direction over the potential range between  $-40$  and  $-90$  mV, but in the inward direction positive to  $-20$  mV (cf. Dudel et al., 1966). The effects were quite reversible.

Panel B shows the voltage dependence of the calcium effect. A difference  $I/V$  relationship (open triangles) was obtained by averaging the data in A for each calcium concentration, and then subtracting the 1.8 Ca value from the 7.2 Ca

value. Data more positive than  $-40$  mV were omitted because they were undoubtedly dominated by changes in steady-state calcium current (see Discussion). The difference  $I/V$  relationship shows a negative slope between  $-70$  and  $-40$  mV. Negative slope was found in the difference  $I/V$  relationship in each of four preparations; the appearance of *net* negative slope in the  $7.2$  mM  $Ca_o$  current voltage relationship was not so consistent (cf. panel A and Fig. 6) but this may be explained by small variations in other currents or in the sealing around the microelectrodes.

Since inward rectifier currents are very sensitive to potassium concentration, the Ca-dependent change was redetermined with  $K_o = 16$  mM (filled triangles). These data were obtained in the same preparation by raising the potassium concentration, and then keeping  $K_o$  constant while carrying out runs in  $7.2$ ,  $1.8$ , and  $7.2$  mM  $Ca_o$  as in A. The overall current-voltage relationships (not shown) were qualitatively similar to those obtained in  $4$  mM  $K_o$ , but they showed very large inward currents negative to  $-60$  mV, as expected from previous studies (e.g. Dudel et al., 1967 *b*). The large inward currents restricted the potential range that could be adequately studied.

Nevertheless, the results in  $16$  mM  $K_o$  demonstrate three important features of the calcium effect. (*a*) The difference  $I/V$  relationship reverses at negative potentials, (*b*) it shows inward rectification, and (*c*) it crosses the  $4$  mM  $K_o$  curve. Each of these features is characteristic of the inwardly rectifying potassium channels in Purkinje fibers. The strongest argument for a change in K conductance is the finding of a reversal potential of the calcium effect in  $16$  mM  $K_o$ . The value of the reversal potential,  $-64$  mV, is reasonably close to  $-59$  mV, the Nernst potential calculated by assuming that  $K_i = 151$  mM and  $K_o = 16$  mM. The  $E_K$  value is only a rough guess since the activity of internal potassium in Purkinje fibers is not known (cf. Lee and Fozzard, 1975) and since the cleft K concentration may be less than  $K_o$  (Cohen et al., 1976). Reversal of the  $4$  mM  $K_o$  difference  $I/V$  relationship would have been expected near  $-100$  mV or so. We did not test this prediction since we were anxious to avoid very large clamp steps which sometimes lead to damage at the current electrode impalement site.

#### *Calcium Does not Change $\bar{i}_{K_2}$*

There are two pathways for potassium ions which show characteristics *a-c* above and which therefore might be enhanced by Ca-rich solutions:  $i_{K_1}$ , the background K current, which is effectively time independent, and  $i_{K_2}$ , the pacemaker potassium current, which displays slow time dependence due to Hodgkin-Huxley-type gating. The question remains, which of these components is augmented by Ca ions? The effect of Ca ions on  $i_{K_2}$  has already been examined in voltage clamp experiments (Brown, 1973; Noble and Tsien, unpublished). These studies showed that calcium ions did not greatly influence the current carried by fully open  $i_{K_2}$  channels. The main effect of calcium-rich solutions was to displace the voltage dependence of channel opening toward less negative potentials. Since these studies were not chiefly concerned with the magnitude of the fully activated current, we have reinvestigated the effect of  $Ca_o$  on  $i_{K_2}$ . Our results confirm the impression that  $i_{K_2}$  is not enhanced by calcium ions.

Fig. 8 illustrates activation curves using the approach of Noble and Tsien (1968). A series of clamp pulses were applied from the holding potential ( $-76$  mV) to various potential levels indicated by the abscissa. The pulses were long enough to allow  $i_{K_2}$  to reach a steady state. Each point gives the level of current after a clamp pulse, soon after returning the potential to  $-76$  mV. The ordinate therefore represents (holding current at  $-76$  mV) + (tail of  $i_{K_2}$  current at  $-76$  mV). The average holding current at  $-76$  mV is given by the horizontal lines, and the  $i_{K_2}$  tails are plotted relative to those levels. This procedure allows the

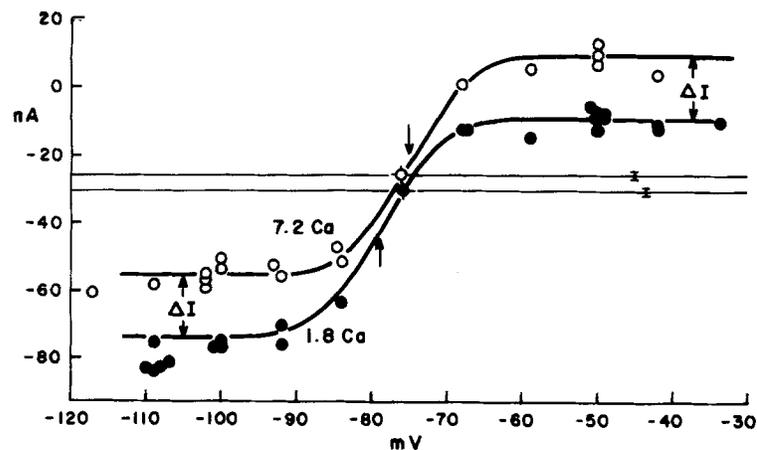


FIGURE 8. Effect of Ca on membrane currents at  $-76$  mV with and without  $i_{K_2}$ . (Abscissa) Voltage level of long clamp pulse. (Ordinate) Net membrane current measured at  $-76$  mV immediately after "off" of clamp pulse. Depolarizing clamps were used to activate  $i_{K_2}$ , and hyperpolarizing pulses to deactivate it. Individual points were obtained by measuring amplitudes of  $i_{K_2}$  tails and plotting them relative to the average holding current during the entire run. Average holding current  $\pm$  SEM are indicated by the horizontal lines and vertical error bars. This procedure reduces scatter due to slow nonprogressive variations in holding current. Smooth curves are generated by the equation  $i = i_{\text{background}} + \bar{i}_{K_2} / [1 + \exp(-(V - V_{1/2})/k)]$ , where  $\bar{i}_{K_2} = 65$  nA and  $k = 4$  mV. The parameters  $i_{\text{background}}$  and  $V_{1/2}$  were adjusted to fit the results in 7.2 and 1.8 mM  $\text{Ca}_o$ . The arrows indicate  $V_{1/2}$ .  $\Delta I$  shows the adjustment in  $i_{\text{background}}$ . Preparation 163-1, apparent cylindrical area  $0.013$  cm $^2$ .

evaluation of the current carried by the fully open  $i_{K_2}$  channels at the holding potential. Immediately after depolarizations to  $-60$  mV or so, the current at  $-76$  mV includes the contribution of all the  $i_{K_2}$  channels; no additional outward current is produced by further depolarizations. Conversely, just after hyperpolarizations to  $-90$  mV or more negative, the current at  $-76$  mV includes no contribution from  $i_{K_2}$ . Thus, the current carried by the fully open  $i_{K_2}$  channels (designated  $\bar{i}_{K_2}$ ) is indicated by the amplitude of the S-shaped relationship.

The data in Fig. 8 were obtained in consecutive runs in 7.2 and 1.8 mM  $\text{Ca}_o$ . A third run in 7.2 mM  $\text{Ca}_o$  showed good recovery but was omitted for simplicity. Both families of points were fitted by the same theoretical curve (see legend)

with a vertical amplitude of about 60 nA. It is apparent then that  $\bar{i}_{K_2}$  remains unchanged by the variation in  $Ca_o$ .

In fitting the points in calcium-rich solution, the smooth curve was displaced upward and to the right relative to its position for the 1.8 mM  $Ca_o$  data. The horizontal displacement is evident from the arrows, which mark the midpoint of the theoretical curve. The voltage shift, 4 mV, is not very different from previously obtained values (Brown, 1973). The vertical displacement (labeled  $\Delta I$ ) reflects the influence of  $Ca_o$  on the  $I/V$  relationship (Figs. 6 and 7). The magnitude of the displacement,  $1.3 \mu A/cm^2$ , agrees well with values obtained in other experiments (Table I). Since  $\Delta I$  can be observed even when  $i_{K_2}$  is fully deactivated (leftmost points) we conclude that the increase in outward current must be due to  $i_{K_1}$  and not  $i_{K_2}$ .

The effect of calcium ions in Fig. 8 may be directly compared with similar analysis of the action of epinephrine (Tsien, 1974, Fig. 6a). Both agents produce depolarizing voltage shifts without altering  $\bar{i}_{K_2}$ . However, epinephrine does not significantly alter the  $i_{K_2}$ -free background current. The simplest interpretation is that unlike Ca ions, epinephrine fails to enhance  $i_{K_1}$ .

#### DISCUSSION

##### *Mechanisms for Ca Control of Action Potential Duration*

The results in this paper reveal two ways in which calcium ions act to shorten the action potential in Purkinje fibers. First, by carrying inward current through the slow inward channel, Ca ions help raise the level of the plateau. This level is important in turn in controlling the speed of time-dependent processes which eventually trigger repolarization. The higher the plateau, the earlier the action potential repolarizes. Second, calcium-rich solutions also favor an earlier repolarization by increasing the background potassium current,  $i_{K_1}$ . A third possibility was considered and ruled out: calcium ions do not favor the onset of delayed K current ( $i_x$ ), as in the case of epinephrine (Tsien et al., 1972). Conversely, epinephrine does not enhance  $i_{K_1}$ . Thus, calcium ions and epinephrine act quite differently on repolarization currents, even though they produce rather similar effects on the height and duration of the action potential in most cases. This reinforces the idea that the action potential may not be a reliable indicator in unraveling ionic mechanisms.

The relative importance of the two mechanisms probably varies from fiber to fiber. Enhancement of  $i_{K_1}$  is the only available explanation on those cases where the plateau is not elevated (Dudel et al., 1966; Temte and Davis, 1967; Fig. 5). In preparations where the plateau is raised by high  $Ca_o$  (the usual finding in our experiments), both mechanisms may come into play. The response of the plateau to calcium-rich solutions shows the same variability found in earlier voltage clamp results (compare Dudel et al., 1966 and Reuter, 1967). Here it is helpful to recognize that calcium ions have major and opposing effects on late currents positive to  $-40$  mV, favoring inward current by enhancing steady Ca influx through the slow inward channels, but enhancing outward current by augmenting  $i_{K_1}$ . The net change may depend on the balance between these actions.

*Increase in  $i_{K1}$* 

Several recent communications have suggested that Ca may control potassium conductance in heart cells (McGuigan and Bassingthwaighte, 1974; Fry et al., 1975; Isenberg, 1975; Colatsky and Hogan, 1975). These studies have raised the intriguing possibility of an internal site of Ca action (see below) but have not focused on the ionic nature of the net current change. In our opinion, the evidence has not clearly indicated an increase in  $g_K$  rather than a decrease in steady  $g_{Na}$  as suggested previously by Dudel et al. (1966).

The question of ionic basis was approached in our experiments by determining the voltage dependence of the Ca effect, and by varying  $K_o$ . The results indicated that the Ca-sensitive pathway is, in fact, a potassium current. The pathway was identified as  $i_{K1}$ , the background K current (inward rectifier). Finding that  $i_{K1}$  is Ca sensitive is particularly interesting because Weld and Bigger (1976) have observed that  $i_{K1}$  is increased by lidocaine. It remains to be seen whether calcium ions and lidocaine have similarities in their modes of action (cf. Weidmann, 1955 *b*).

Inhibitors of the inward rectifier are well known in skeletal muscle (Adrian, 1964; Stanfield, 1970) but we are unaware of an agent which enhances this component. Large increases in  $g_K$  occur in fatigued skeletal muscle fibers and may depend on calcium (for review, see Lüttgau, 1974). It will be interesting to learn if the inward rectifier is involved.

*Site of Ca Action*

Our studies focused on the ionic basis of the Ca effect, and were less concerned with where the Ca ions act. It would be desirable, of course, to know if Ca ions can influence  $i_{K1}$  from the outside, or if they must first enter the cell. The possibility of an internal site of action is attractive since it might explain why agents which elevate  $Ca_i$  (metabolic inhibitors, cardiac glycosides) also shorten the action potential (see McGuigan and Bassingthwaighte, 1974; Isenberg, 1975). It might also explain why Ca antagonists like Mn ions or D600 lengthen the action potential at certain concentrations (Kass and Tsien, 1975; Colatsky and Hogan, 1975).

We have tested the hypothesis that the increase in  $i_{K1}$  is mediated by Ca entry through the slow inward channel. Table I summarizes the results. Ca-dependent displacements in membrane current were determined using various procedures. One method (designated *P* in Table I and illustrated in Fig. 6) used a standard depolarizing prepulse to evoke the slow inward current. The clamp pulses were applied at the same rate used for driving action potentials. Other procedures deliberately avoided slow inward current activation by omitting the depolarizing prepulse. The membrane potential was either stepped directly from a holding potential of  $-50$  mV to more negative levels (*N*) or it was changed in a slow "voltage staircase" (see Hauswirth et al., 1969, Fig. 3) which allowed currents to achieve steady-state levels (*S*). Each of these procedures gave similar values. There was no obvious enhancement of the  $i_{K1}$  increase with slow inward current activation at clamp rates up to 60/min over a period of 2 min. In all cases, the  $\Delta I/V$  relation showed inward rectification with negative slope similar to the 4 mM  $K_o$

TABLE I  
Ca-DEPENDENT DISPLACEMENT OF MEMBRANE CURRENT

Preparation	Method	Ca <sub>o</sub> , high/low	V	ΔI
			mV	μA/cm <sup>2</sup>
120-1	S	7.2/1.8	-69	1.92*
163-1	S	7.2/1.8	-76	1.34
162-1	S	5.4/1.8	-68	1.14*
162-1	P	7.2/1.8	-68	1.22*
160-2	P	7.2/0.9	-70	2.56
163-2	P	7.2/1.8	-68	1.07
163-2	N	7.2/1.8	-68	0.81
Average:				1.44

\* Average value using bracketing runs in calcium-rich solution.

S = steady-state current.

P = current at V after prepulse which activated slow inward current (see Fig. 6).

Clamps applied at 60/min in 163-2, otherwise at 20/min.

N = no prepulse.

curve in Fig. 7. Near  $-70$  mV, the average  $\Delta I$  was  $1.44 \mu\text{A}/\text{cm}^2$ . The tentative conclusion is that slow inward current activation may not be very important in the Ca-dependent increase in  $i_{K1}$ . The question of an internal site of action remains unsettled, of course, since Ca may enter the cell via other pathways such as Ca-Na exchange (Reuter and Seitz, 1968).

#### *Different Repolarization Processes in Purkinje Fiber and Ventricular Muscle?*

Our experiments showed that the Purkinje fiber action potential can be curtailed by artificially elevating the plateau level. This result is worth discussing independently, because it provides basic information on the nature of repolarization without resorting to voltage clamp. It argues against the idea that repolarization is merely the recharging of membrane capacity by a nonlinear, time-independent outward current (Kootsey and Johnson, 1973). If this were the case, raising the plateau level could only delay the final repolarization. The observed behavior reflects the importance of repolarization current(s) showing time dependence as well as voltage dependence. Thus, the experiments support conclusions reached previously through studies of all-or-nothing repolarization (Vassalle, 1966) or measurements of membrane current under voltage clamp (see Noble and Tsien, 1972 for review).

Ventricular muscle differs from Purkinje fiber tissue in its response to imposed potential changes during the plateau. Brief intracellular current pulses have been applied to a variety of myocardial preparations (Cranefield and Hoffman, 1958; Sakamoto, 1969; Šumbera, 1970). In general, imposed depolarization during the plateau did not decrease the action potential duration. One explanation for the difference is that Purkinje fibers display prominent delayed rectification over the plateau range due to  $i_x$  (Noble and Tsien, 1969) whereas most ventricular preparations show little slow outward current (Giebisch and Weidmann, 1971; McGuigan, 1974; cf. Ochi, 1970). Alternatively, it seems possible that the level of the ventricular plateau is high enough in low Ca<sub>o</sub> to

keep voltage-dependent variables close to saturation. Finally, there may be a contrast in the properties of the slow inward current. In mammalian ventricle, calcium current inactivation becomes slower as the membrane potential becomes more positive (Beeler and Reuter, 1970; Trautwein et al., 1975). This would be in the wrong direction for a higher plateau to produce an earlier repolarization. However, the voltage dependence of slow inward current inactivation may be different in Purkinje fibers. Hiraoka and Fozzard (in preparation) found that depolarization accelerates inactivation by decreasing  $\tau_f$  over the range between  $-60$  and  $-30$  mV. Their results are consistent with the action potential model used in this paper to simulate the effects of applied current pulses. The resemblance between calculation (Fig. 4 C) and experiment (Fig. 4 A, B) is encouraging, but we still need more information on slow inward current inactivation positive to  $-30$  mV. A full understanding of the influence of plateau level on action potential duration will help clarify the differences between action potential mechanisms in Purkinje fiber and ventricular muscle.

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