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# EFFECTS OF READMISSION OF SUBSTRATE ON THE MEMBRANE POTENTIAL IN GLYCOGEN-DEPLETED GUINEA-PIG TAENIA COLI

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## SUMMARY

1. In the glycogen-depleted smooth muscle of the guinea-pig taenia coli, application of glucose or $\beta$-hydroxybutyrate ($\beta$-HB) in the presence of 20 mM-K or carbachol (5 $\mu$M) produced a transient hyperpolarization for about 1 min followed by a sustained depolarization accompanied by spike activity.

2. The early hyperpolarization was highly temperature dependent, so that below 30 °C, substrate application produced the depolarization with a delay of approximately 2 min, without a clear preceding hyperpolarization.

3. The responses to substrate were not affected by ouabain (10 $\mu$M). Readmission of K after a treatment with K-free solution for 10 min caused a transient hyperpolarization only in the presence of substrate. This hyperpolarization was abolished by ouabain.

4. Verapamil (0·1 $\mu$M) blocked the substrate-induced depolarization, revealing an underlying slow hyperpolarization. Removal of Ca abolished both the hyperpolarization and depolarization caused by substrate application, but the hyperpolarization disappeared much more slowly than the depolarization.

5. Removal of the external K had little effect on the substrate-induced hyperpolarization, but the hyperpolarization induced by substrate application was increased when the external K was increased up to approximately 20 mM. Both the hyperpolarization and the depolarization were not clearly affected by completely replacing Na with choline.

6. The results suggest that ATP supplied by the addition of substrate activates some electrogenic pump, probably a Ca pump, causing hyperpolarization, and that ATP also removes the inactivation of Ca conductance with some delay, resulting in an increased depolarization.

## INTRODUCTION

In a previous paper it has been shown, in glycogen-depleted preparations of the guinea-pig taenia coli, that readmission of a substrate during the K contracture produces an increase in tension development preceded by a transient relaxation (Ashoori, Takai, Tokuno & Tomita, 1984). In similar conditions, an increase of the K depolarization and spike activity was observed when the substrate was readmitted.

These results were explained by a hypothesis that ATP produced by substrate readmission reactivates Ca channels which have been inactivated, resulting in an increase of Ca influx. Thus, metabolism is probably controlling not only the contractile machinery but also the permeability of the plasma membrane through ATP supply.

No clear change in membrane potential was previously seen corresponding with the early relaxation following substrate readmission. During further experiments, we noticed that the measurement of membrane potential was made at a temperature which was about 5 °C lower than that in the organ bath used for tension recording (35 °C). When the temperature was adjusted to 35 °C, a clear transient hyperpolarization always appeared before the depolarization following substrate readmission. Thus, there is a good correlation between the mechanical responses and changes in membrane potential.

In the present experiments, the factors underlying the electrical responses, both the early transient hyperpolarization and the following continuous depolarization, caused by readmission of glucose or $\beta$-hydroxybutyrate were further investigated in glycogen-depleted guinea-pig taenia coli. The results support the previous hypothesis that intracellular ATP controls Ca channels of the plasma membrane and, in addition, they suggest that the transient hyperpolarization caused by substrate readmission is due to activation of an electrogenic Ca pump.

## METHODS

The methods used were the same as previously described (Ashoori *et al.* 1984). Guinea-pigs of either sex were used after stunning and bleeding. Small pieces of the taenia coli were isolated. The size of the preparations was slightly larger than those used for the previous experiments, being 100–150 × 100–150 $\mu$m. In order to deplete glycogen, the preparations were pre-treated with glucose-free, Ca-free, excess-K (126 mM) solution containing 20 mM-Na, and Ca was readmitted four times for 20 min at intervals of 30 min. The glycogen content of the preparation after this treatment was expected to be less than 3% of the control, based on previous results.

Micro-electrodes were filled with 2·5 M-KCl solution and had a resistance ranging between 35 and 50 MΩ. Continuous recording for more than 1 h from a single fibre was often possible since movement of the preparation was reduced by pinning it down with many fine tungsten wires of 15 $\mu$m diameter. The records shown in the Figures were all obtained from successful continuous recordings. The indifferent electrode was an Ag–AgCl electrode coated with agar–agar in a glass tube of 2 mm inside diameter. The chamber contained 0·5 ml solution and it was continuously perfused at a rate of 3 ml/min with solutions warmed to 35 °C.

The normal glucose-free solution had the following composition (mM): NaCl, 143; $KHCO_3$, 5·9; $CaCl_2$, 2·4; $MgCl_2$, 1·2, bubbled with a gas mixture of 97% $O_2$ and 3% $CO_2$. When a substrate was applied, or the ionic composition was modified, the osmolarity was always compensated by changing the NaCl concentration. When Ca was removed, the Mg concentration was increased to 12 mM to prevent depolarization, and 0·5 mM-EGTA (ethyleneglycol-bis-($\beta$-aminoethyl ether) *N,N,N',N'*-tetraacetic acid) was added.

## RESULTS

All preparations were depleted of glycogen as described in the Methods section. The membrane potential of glycogen-depleted preparations was between −55 and −60 mV. These values were within the range observed in normal conditions (cf. Kuriyama, 1963). However, spontaneous spike activity was usually not observed.

When the external K concentration was increased to 20 mM, the membrane was depolarized to slightly more than 20 mV, this being accompanied by spike activity. The spike activity disappeared within 1–2 min, the rate of disappearance varying slightly between different preparations. Fig. 1 shows the effect of β-hydroxybutyrate (β-HB, 11·8 mM) applied during the depolarization by 20 mM-K. At 35 °C, β-HB

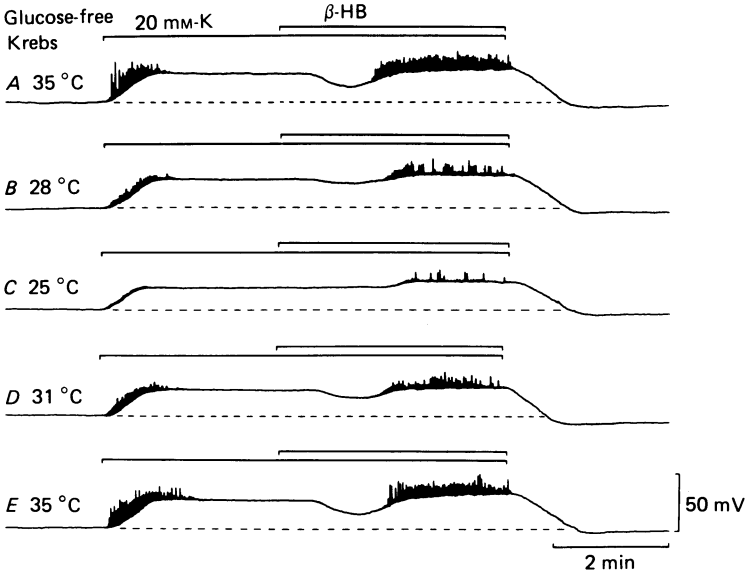


Fig. 1. Effects of temperature on the changes in membrane potential produced by 20 mM-K and β-hydroxybutyrate (β-HB, 11·8 mM) in the presence of 20 mM-K. Continuous intracellular recording from a glycogen-depleted taenia coli. When the temperature was changed, the response to 20 mM-K followed by 11·8 mM-β-HB was observed 10 min after stabilizing the temperature at the new level. This procedure (*A*) was repeated in *B–E* at intervals of 20 min, at different temperatures, as indicated at the beginning of each trace. The application of 20 mM-K and β-HB is indicated above each record. For further explanation, see text.

produced about 10 mV hyperpolarization with a delay of approximately 30 s, reaching the maximum within 30–50 s, as shown in *A*. The hyperpolarization gradually converted to a depolarization of about 5 mV on which spike activity appeared. Thus, this electrical response corresponds well to the mechanical response previously observed (Ashoori *et al.* 1984), which was a transient relaxation followed by an increase in tension when substrate was applied to a glycogen-depleted preparation. When the temperature was lowered to 28 °C (*B*), the degree of hyperpolarization was significantly reduced and at 25 °C (*C*), the hyperpolarization disappeared. The amplitude of spikes superimposed on the depolarization decreased with cooling. The depolarization caused by β-HB was less temperature dependent than the preceding hyperpolarization, so that, at 25 °C, β-HB produced only depolarization, but its latency was nearly the same as the duration of the early transient hyperpolarization at 35 °C. The effect of cooling was reversible, as shown in *D* and *E*. All the subsequent experiments were carried out at 35 °C.

An experiment with the same procedure was performed in the presence of verapamil (Fig. 2). As previously shown (Ashoori *et al.* 1984), verapamil (0·1 $\mu$M) blocked the spike activity and the depolarization caused by substrate readmission, revealing the underlying hyperpolarization clearly (*A*). The degree and time course of the early part of the hyperpolarization was not affected by verapamil. When $\beta$-HB

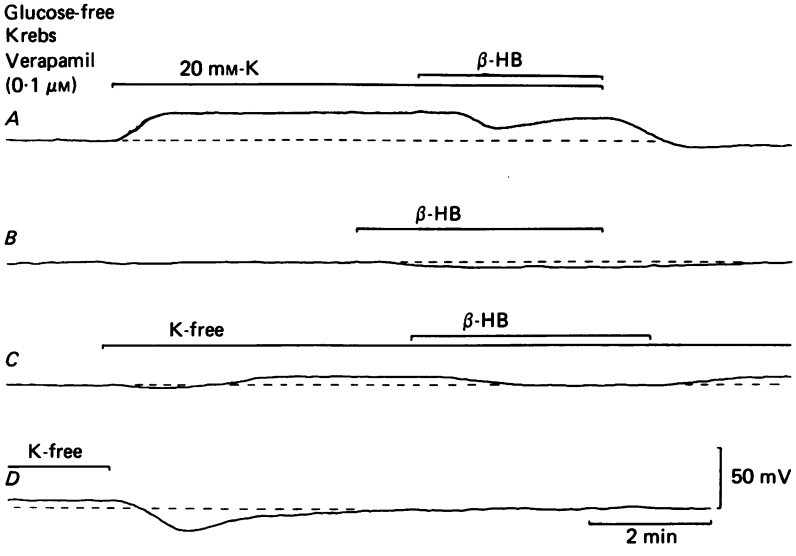


Fig. 2. Effects of $\beta$-HB application at 20, 5·9 and 0 mM-K, and of K removal and K readmission in the presence of verapamil, continuous record from the same cell. *A*, response to $\beta$-HB (11·8 mM) in the presence of 20 mM-K; *B*, response to $\beta$-HB in the presence of 5·9 mM-K; *C*, K removal and $\beta$-HB application in the absence of K; *D*, response to K readmission 13 min after K removal and 4 min after wash-out of $\beta$-HB. Note the similarity between hyperpolarization caused by $\beta$-HB in the presence of 20 mM-K (*A*) and by K (5·9 mM) readmission 4 min after treatment with $\beta$-HB for 4 min. Also note that the effect of $\beta$-HB application was smaller and slower in 5·9 mM-K (*B*) than in 20 mM-K (*A*), but it was not much affected by removal of K (*C*).

was readmitted at 5·9 mM-K, it produced a smaller and slower hyperpolarization compared with the change in membrane potential at 20 mM-K (*B*). The effect of $\beta$-HB in the absence of K (*C*) was also not significantly different from that in the presence of 5·9 mM-K. In *D*, K was readmitted after treatment with K-free solution for 13 min and 4 min after removal of $\beta$-HB, and this produced a large transient hyperpolarization. However, as the interval between the removal of $\beta$-HB and K readmission was prolonged the hyperpolarization caused by K readmission became smaller and nearly disappeared when the interval was about 20 min. Similarly, K readmission did not produce any significant hyperpolarization when K readmission was not preceded by substrate readmission.

The hyperpolarization induced by K readmission following $\beta$-HB application is likely to be due to activation of an electrogenic Na pump, as shown previously in this tissue (Casteels, Droogmans & Hendrickx, 1971; Tomita & Yamamoto, 1971). The time course of hyperpolarization was similar in the responses to $\beta$-HB readmission

in the presence of 20 mM-K (Fig. 2*A*) and to K readmission following β-HB treatment (Fig. 2*D*), suggesting that Na-pump activity may be responsible for both responses.

Fig. 3 shows the effects of ouabain on substrate readmission during 20 mM-K depolarization. Readmission of β-HB and glucose produced much the same responses and no apparent change was observed in the presence of ouabain (10 μM). On the other

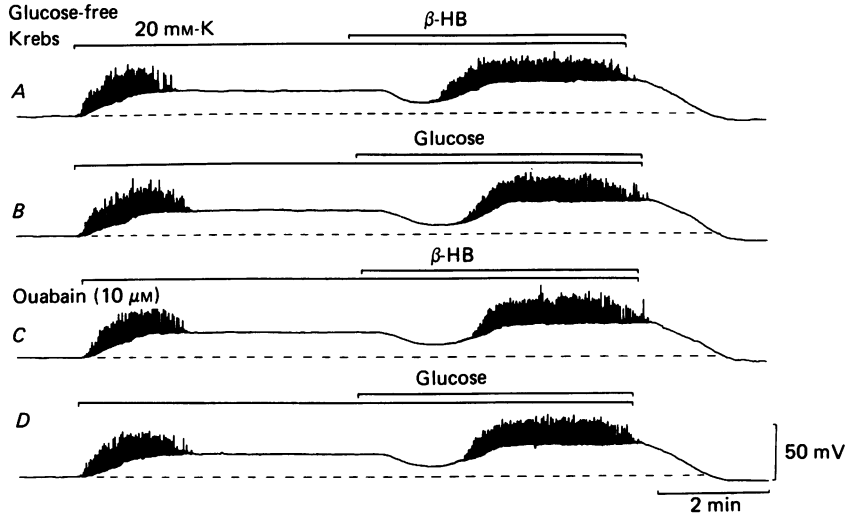


Fig. 3. Effects of ouabain (10 μM) on the responses to β-HB (11·8 mM) or glucose (11·8 mM) application. Addition of β-HB (*A*) and glucose (*B*) during the depolarization by 20 mM-K before, and similar responses (*C* and *D*) after ouabain application. Responses were obtained at 20 min intervals as a continuous record. Ouabain was added 10 min before application of 20 mM-K in (*C*). Note that ouabain had no significant effect on the responses to 20 mM-K and substrate readmission.

hand, the hyperpolarization due to K readmission was readily abolished by ouabain, confirming the previous observation (Casteels *et al.* 1971; Tomita & Yamamoto, 1971). Thus, although the hyperpolarization produced by substrate readmission was similar to that produced by K readmission in the presence of a substrate, the underlying mechanisms for these responses were clearly different.

It has been shown that substrate readmission increases the tissue content of high-energy phosphate compounds (ATP and creatine phosphate) (Ashoori *et al.* 1984). However, it is not clear whether these compounds are acting directly on the plasma membrane or indirectly after being converted to cyclic AMP. In the experiment shown in Fig. 4, the effects of dibutyryl cyclic AMP and theophylline, a phosphodiesterase inhibitor, were studied. The application of dibutyryl cyclic AMP (0·5 mM) for 7 min had no clear effect on the depolarization produced by 20 mM-K (*B*). In another experiment, the preparation was treated with dibutyryl cyclic AMP (0·5 mM) for 10 min before depolarization by 20 mM-K, but no appreciable change in the depolarization was observed. On the other hand, theophylline (10 mM) markedly repolarized the membrane, but after removal of theophylline the membrane was depolarized again to the same level as before its application (*C*). A potentiation of

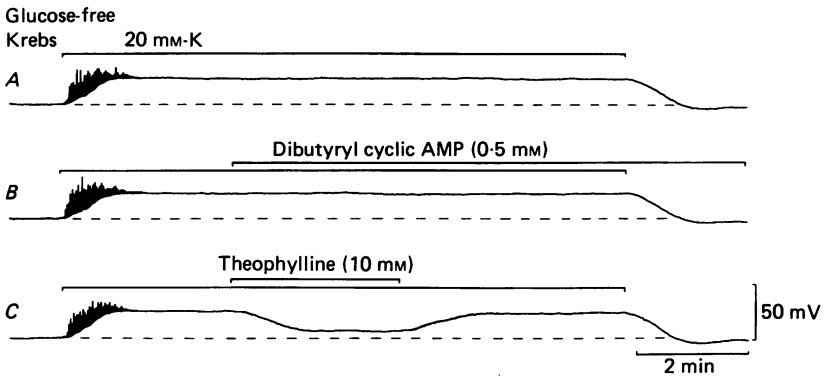


Fig. 4. Effects of dibutyryl cyclic AMP and theophylline applied during the depolarization produced by 20 mM-K applied at 20 min intervals. *A*, control. Note that dibutyryl cyclic AMP (0·5 mM) had no effect (*B*), but that theophylline (10 mM) repolarized the membrane without affecting the depolarization after its removal (*C*).

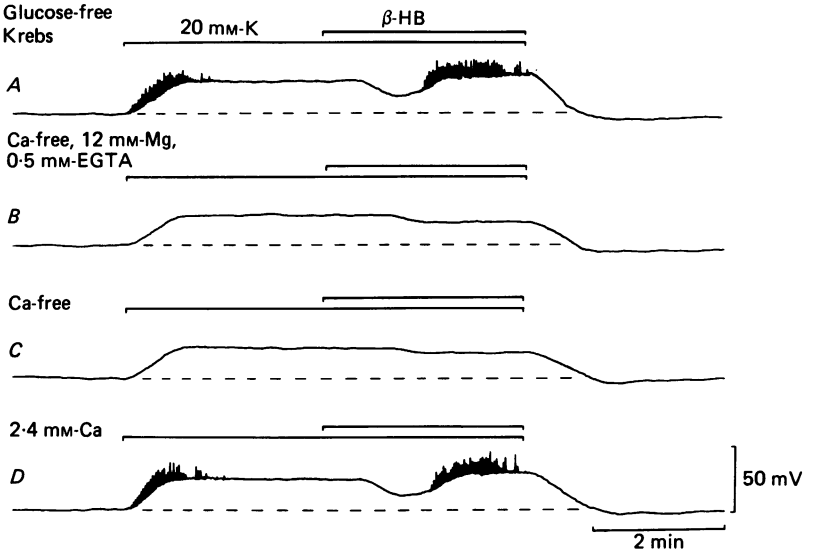


Fig. 5. Effects of Ca removal on the response to β-HB application. When Ca was removed, Mg was increased to 12 mM and 0·5 mM-EGTA was added. Responses to 20 mM-K and β-HB: *A*, control; *B*, 10 min; and *C*, 36 min after Ca removal. *D*, recovery 10 min after readmission of normal Ca (2·4 mM). Note abolition of depolarization and slow disappearance of hyperpolarization caused by β-HB application.

the K depolarization, as seen with substrate readmission, was never observed. These results suggest that the response to substrate readmission is probably mediated by ATP itself, not by cyclic AMP. The mechanism of hyperpolarization caused by theophylline has not been investigated in the present experiments.

Since verapamil blocked the depolarization phase of the response to substrate readmission, the depolarization may be the result of an increase in Ca conductance, as previously proposed (Ashoori *et al.* 1984). To confirm this, the effects of Ca removal

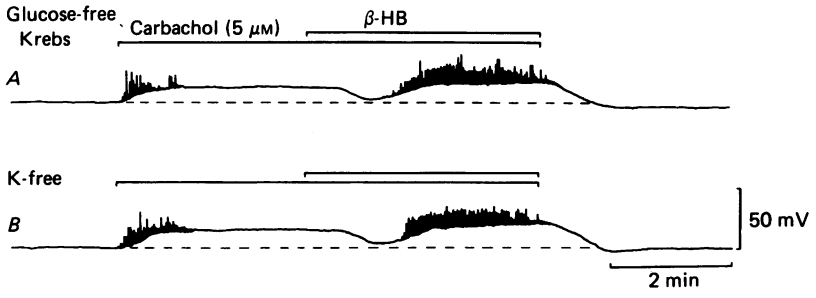


Fig. 6. Effects of K removal on the response to β-HB (11·8 mM) in the presence of carbachol (5 μM). *A*, control response to carbachol and β-HB. *B*, responses obtained 10 min after removal of K. Note the weak effect of K removal on the responses to carbachol and β-HB.

were studied, as shown in Fig. 5. After observing the control response to β-HB during depolarization by 20 mM-K (*A*), Ca was removed, 0·5 mM-EGTA was added, and Mg was increased to 12 mM. 7 min later, K was increased to 20 mM again and β-HB was readmitted (*B*). After an interval of 20 min in Ca-free solution the same experiment was repeated (*C*). The depolarization caused by 20 mM-K was not significantly affected, but the depolarization and spike activity evoked by β-HB were completely abolished, as expected from the experiment with verapamil. The hyperpolarization produced by β-HB was suppressed increasingly with the time of exposure to Ca-free solution. The recovery of the response 10 min after returning to 2·4 mM-Ca was perfect (*D*). When the external Mg concentration was increased to 12 mM in the presence of 2·4 mM-Ca, the spike activity was partially suppressed, but the transient hyperpolarization and following depolarization induced by substrate application were not apparently modified for at least 40 min. Thus, the effect shown in Fig. 5 was considered to be due to Ca removal, but not to excess Mg.

As already shown in Fig. 2, the hyperpolarization produced by β-HB application at 5·9 mM-K was similar in K-free solution. This lack of effect of K removal was also observed on the hyperpolarization by β-HB during depolarization caused by carbachol (5 μM), as shown in Fig. 6. When β-HB was applied during carbachol depolarization, it produced a transient hyperpolarization followed by a depolarization (*A*) which were very similar to those observed in 20 mM-K. Both the hyperpolarization and also the depolarization in response to β-HB in the presence of carbachol were nearly identical in the presence of 5·9 mM-K (*A*) and in the absence of K (*B*).

In Fig. 7, glucose was readmitted without depolarizing the membrane, as shown in Fig. 2*B* with β-HB, but in the absence of verapamil. The time course and the degree of hyperpolarization induced by glucose were similar to those induced by β-HB in the presence of verapamil (Fig. 2*B*). However, in the absence of verapamil, spontaneous spike activity of a large amplitude appeared with a delay of more than 5 min after glucose application. Since the hyperpolarization gradually declined, the level of the membrane potential at which the spontaneous spike activity started was nearly the same as before glucose readmission. The appearance of spike activity was similarly observed with readmission of β-HB. Thus, the hyperpolarization induced by substrate readmission was much weaker at 5·9 mM-K than at 20 mM-K, independent of verapamil.


Fig. 7. Effects of glucose readmission to glucose-free Krebs solution (5·9 mM-K) in glycogen-depleted preparations. *A* and *B*, different preparations. Note that glucose application produced a small and slow hyperpolarization and that the spike activity appeared after a much longer delay compared with the response observed in the presence of 20 mM-K. Also note that the spike amplitude was larger than in 20 mM-K.

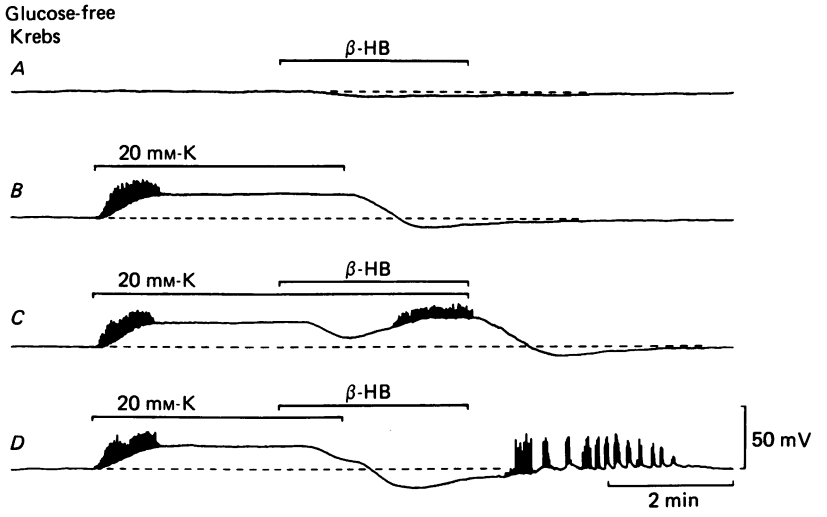


Fig. 8. Responses to β-HB (11·8 mM) at 5·9 and 20 mM-K. *A* and *B*, responses to β-HB and 20 mM-K alone, respectively. *C*, β-HB response in 20 mM-K solution; and *D*, recovery from excess K, 1 min after β-HB application. Each record was obtained at 20 min intervals. Note that reduction of K concentration from 20 to 5·9 mM in the presence of β-HB produced a large hyperpolarization (*D*).

Fig. 8 shows the effects of β-HB application in relation to 20 mM-K treatment. At 5·9 mM-K, an application of β-HB to a glycogen-depleted preparation produced a small (less than 5 mV) hyperpolarization (*A*), as already shown in Fig. 2*B*. When the external K concentration was increased to 20 mM, the membrane was depolarized by about 20 mV, accompanied by a transient spike activity in the absence of substrate. During the recovery from excess-K treatment, there was always a phase of slow hyperpolarization of 5–10 mV (*B*). A typical transient hyperpolarization of more than 10 mV was observed on β-HB application during the depolarization by 20 mM-K (*C*).

In (*D*), the K concentration was reduced from 20 to 5·9 mM, 1 min after application of β-HB, coinciding with the hyperpolarization due to the effect of β-HB. The reduction of the K concentration in the presence of β-HB hyperpolarized the membrane by more than 15 mV, which was larger than the substrate-induced hyperpolarization observed at 5·9 (*A*) or 20 mM-K (*C*).

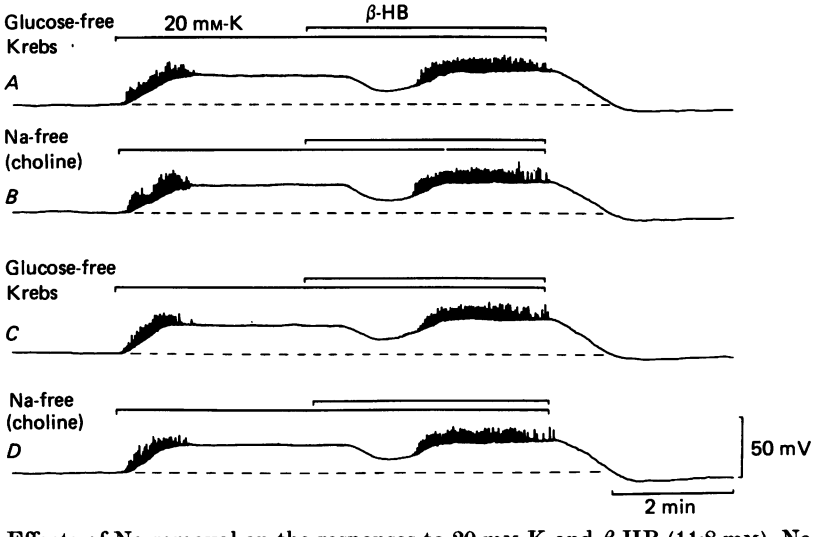


Fig. 9. Effects of Na removal on the responses to 20 mM-K and β-HB (11·8 mM). Na was completely replaced with choline and atropine (10 μM) was added to all solutions throughout the experiment. *A*, control; *B*, 10 min after Na removal as a continuous record. *C* and *D*, the same experiment as *A* and *B*, but obtained from another preparation. Note no effect of Na removal on the responses.

The responses to β-HB application were not affected by removal of the external Na, as shown in Fig. 9. In this Figure, examples obtained from two different preparations are shown. After observing the control response to β-HB during 20 mM-K depolarization (*A* and *C*), Na was completely replaced isosmotically with choline, and 10 min later in *B* and 15 min later in *D*, the same experiment was repeated in the absence of Na. The responses were nearly identical in the presence (*A* and *C*) and absence of Na (*B* and *D*).

## DISCUSSION

In a previous paper, it has been shown that in the glycogen-depleted taenia coli, glucose or β-HB application increases the Ca-induced contraction, oxygen consumption and intracellular high-energy phosphate compounds in glucose-free, excess-K solution (Ashoori *et al.* 1984). Since substrate application increases the depolarization caused by 20 mM-K or 1 μM-carbachol, and this depolarization is blocked by verapamil, the increase in tension was interpreted as being partly due to an increase in Ca conductance of the plasma membrane caused by ATP supply.

In the present experiments, carried out at 35 °C, it was found that substrate readmission during the depolarization by 20 mM-K produced a biphasic response: a

transient hyperpolarization preceding an increase in the sustained depolarization accompanied by spike activity. Verapamil only blocked the depolarization response, leaving the hyperpolarization intact. An increase in oxygen consumption during substrate application was still clearly observed in the presence of verapamil, although it blocked the increase in tension (unpublished observation). Thus, the hyperpolarization is also likely to be caused by an increase in ATP supply.

The early transient hyperpolarization caused by substrate application does not seem to be the result of an increase in K conductance or a decrease in Na conductance, because the hyperpolarization is not affected by removal of K or Na from the external medium. A contribution of an electrogenic Na pump can also be neglected, because the hyperpolarization is not modified by treatment with 10 $\mu$M-ouabain, which readily abolishes the hyperpolarization due to Na-pump activation caused by K readmission (Casteels *et al.* 1971; Tomita & Yamamoto, 1971).

Therefore, the possibility remains that substrate readmission may activate an electrogenic Ca pump. This idea is in accord with the observation that the hyperpolarization is highly dependent on the temperature, that the hyperpolarization slowly disappears after Ca removal, and also that the tension is decreased during the hyperpolarization as shown in the previous experiments (Ashoori *et al.* 1984). The reason why the hyperpolarization is increased by raising the external K concentration to 24 mM is not clear. However, since the substrate-induced hyperpolarization is greatly potentiated during a process of recovery from excess K (Fig. 8), it may be that the activity of the Ca pump is determined not only by the availability of ATP but also by the concentration of Ca near the intracellular pumping sites, and that the Ca concentration in this compartment is increased by membrane depolarization. When the external K concentration was increased to more than 24 mM, the hyperpolarization was reduced (unpublished observation), probably due to a decrease in membrane resistance.

The maximum hyperpolarization caused by substrate application is about 10 mV in 20 mM-K solution, and this slowly disappears in 2–3 min. If it is assumed that the hyperpolarization is due to an electrogenic Ca pump uncoupled with a counter-ion and that the membrane resistance is 30 k$\Omega$ cm$^2$, the maximum rate of Ca efflux would be 1·5 pmol/cm$^2$ . s, which is ten times larger than that reported for the squid giant axon (DiPolo & Beaugé, 1983). The membrane resistance in 20 mM-K medium is not known, but a value of 30 k$\Omega$ cm$^2$ may be realistic (Tomita, 1970). Thus, it seems possible to produce hyperpolarization of 10 mV by activating a Ca pump. The degree of hyperpolarization is likely to be influenced by many factors, such as the Ca concentration close to the inner surface of the plasma membrane, the availability of ATP, and the membrane resistance.

The hyperpolarization induced by substrate application observed in the present experiments seems to be the same process as that responsible for suppression of the spontaneous spike activity with some hyperpolarization reported previously as a response to glucose readmission following simple glucose removal (Axelsson & Bülbring, 1961; Axelsson, Högberg & Timms, 1965). However, in the present experiments, glycogen depletion was probably much more complete compared with the previous experiments, because simple removal of glucose from the external medium reduces tissue glycogen only very slowly (Bueding & Hawkins, 1964; Axelsson *et al.* 1965; Ashoori *et al.* 1984). The very poor appearance of spontaneous

spike activity in the present experiments may partly be due to the very severe glycogen depletion and probably also to the much smaller size of the preparation.

The depolarization caused by substrate readmission is easily blocked by verapamil and also readily by removal of the external Ca. However, the depolarization and spike activity are not significantly affected by removal of either the external Na or K. The depolarization is less dependent on the temperature than the preceding hyperpolarization. Thus, as previously proposed (Ashoori *et al.* 1984), the depolarization is likely to be due to an increase in Ca conductance of the plasma membrane.

In cardiac muscle fibres, stimulation of the $\beta$-adrenergic receptor is known to increase Ca conductance and this action is mimicked by intracellular cyclic AMP or ATP (Trautwein, Taniguchi & Noma, 1982; Cachelin, De Peyer, Kokubun & Reuter, 1983; Irisawa & Kokubun, 1983; Tsien, 1983). In the present experiments, dibutyryl cyclic AMP had no effect on the membrane potential in the absence of substrate and theophylline did not produce any potentiation of the depolarization caused by 20 mM-K. Thus, in the smooth muscle of the guinea-pig taenia coli, ATP is probably used directly to increase the Ca conductance, not after being converted to cyclic AMP. ATP may be utilized to remove inactivation of the Ca channel by reducing the local Ca concentration near the channel. However, the different effects of temperature on the hyperpolarization and depolarization following substrate application throw some doubt on this explanation, although Ca reuptake into the sarcoplasmic reticulum may still be enough to decrease the free Ca concentration at low temperature. Thus, a direct action of ATP on the Ca channels is proposed as a tentative hypothesis.

In the absence of excess K or carbachol, substrate application produces a weak prolonged hyperpolarization and generates spontaneous spike activity following a gradual disappearance of hyperpolarization taking more than 5 min. On the other hand, in the presence of 20 mM-K or carbachol, the depolarization and spike activity start after a short hyperpolarization of about 1 min. Therefore, ATP does not seem to open Ca channels directly, but ATP is probably activating some process or restoring an inactivated process responsible for excitation in response to excess K and carbachol, or for spontaneous spike activity. It may be that ATP is necessary to maintain Ca channels in an activated state and that the depolarization is increased by substrate application due to removal of inactivation of Ca channels by supplying ATP.

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