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ELECTROPHYSIOLOGY OF THE HEART OF AN ISOPOD CRUSTACEAN: *PORCELLIO DILATATUS*

II. EFFECTS OF IONS AND MEMBRANE PERMEABILITY INHIBITORS

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INTRODUCTION

In a previous publication (Holley & Delaleu, 1972) we described some electro-physiological properties of the heart of an isopod crustacean, the wood-louse *Porcellio dilatatus*, with special reference to the characteristics of the intracellular electrical response and some aspects of the relationship between the latter and the contractile process.

The electrical response of the myocardium to the nerve pace-maker displayed a profile resembling that of the action potential in vertebrate hearts. However, the response height was small (35–45 mV) and had no overshoot. The response was thought to include two components: an early depolarizing phase, mainly synaptic in nature; and a sustained plateau and a repolarization involving weakly active generating mechanisms. When stimulated by intracellular currents the heart membrane revealed weak 'normal' rectifying properties, generated sometimes small graded responses, but did not produce action potentials.

The purpose of this present work is to complete the study of the electrical response by investigating its ionic requirements. This question is of particular interest since we have suggested that the electrical activity of the heart of *Porcellio* probably differs from that of other Crustacea with respect to the nature, active and not purely synaptic, of the sustained depolarization; and that, on the other hand, this phase is effective in controlling contraction.

We exposed the heart of *Porcellio*, when spontaneously active or intracellularly stimulated, to the action of salines containing various concentrations of K^+, Na^+ and Ca^{2+}. In addition, we tested substances known to be inhibitors of specific ionic permeabilities – tetrodotoxin (TTX) which inhibits the Na^+ conductance (Kao, 1966; Moore & Narahashi, 1967), Mn^{2+} which suppresses Ca^{2+}-spikes (Fatt & Ginsborg, 1958; Hagiwara & Nakajima, 1966; McCann, 1971) and blocks the 'slow channel' in vertebrate heart (Rougier *et al.* 1969; Ochi, 1970), and tetraethylammonium (TEA) which reduces the potassium permeability (Hagiwara & Watanabe, 1955; Tasaki & Hagiwara, 1957; Hille, 1967).

Marked changes in electrogenesis having been shown to occur in TEA solution, we played special attention to the mechanisms involved.

MATERIAL AND METHODS

The heart tubes were dissected and isolated as described previously (Holley & Delaleu, 1972). The control bathing medium, i.e. a modified Ringer solution, determined by Holley & Regondaud (1963), comprised the following ions, in mM/l: Na^+, 306·6; K^+, 6; Ca^{2+}, 13·5; Cl^-, 323·7; CO_3H^-, 2·4; pH was 7·6. In Na^+-deficient solutions NaCl was replaced by equimolar amounts of choline chloride. When necessary, pH was adjusted by adding Tris to the bathing saline. The temperature of the bath during an experiment was maintained between 22 and 25 °C. The electrical activity of the heart was recorded with glass intracellular microelectrodes (5–15 MΩ resistance) filled with 3 M potassium chloride. In many experiments the myocardial cells were stimulated intracellularly using a second microelectrode as described by Weidmann (1951). The recording of the heart contraction was made by means of a RCA 5734 transducer.

RESULTS

Role of various ions on the resting and active membrane

Membrane potential and external concentration of potassium

Fig. 1 shows the changes in the resting membrane potential against $[K^+]_0$ on a logarithmic scale (6–120 mM/l). It is only at high ion concentrations that the slope of the curve, which summarizes three experiments, approaches the theoretical value of 58 mV per decade. The results indicate that for potassium concentrations near normal $[K^+]_0$ the heart membrane does not behave like at a highly selective potassium battery.

Effect of potassium on spontaneous electrical responses

Fig. 2(a) shows the effect of excess potassium (24 mM/l) on the electrical activity. First, the membrane was depolarized, as described previously, and the frequency of the responses was slightly increased. Then their amplitude decreased and the maximum upstroke tended to approach the reference potential. The early depolarization was accelerated 60 sec after, whereas at the same time the rapid phase was slowed down. In addition, the rate of fall was increased, reducing the response duration. The value of the membrane potential was 25 mV, and all electrical activity had disappeared 90 sec after the introduction of this modified solution. These phenomena were perfectly reversible.

The removal of K^+ from the bathing solution produced an alteration of the electrical activity as shown in Fig. 2(b). The resting membrane voltage was little affected, and furthermore the direction of its variation was not constant; either it increased by a few mV during the first minute and then progressively decreased (its value was then less than the normal by 5 mV) or it decreased gradually during the entire experiment (9 min). In this case the depolarization never exceeded 6 mV. The rhythmicity was generally increased, whereas the response amplitude decreased by
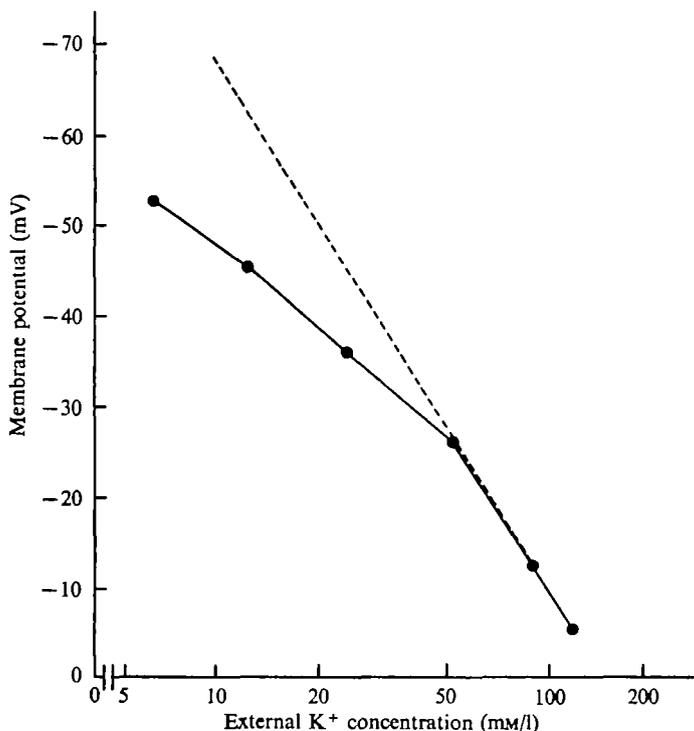
Fig. 1. Resting membrane potential plotted as a function of the external K^+ concentration, on a logarithmic scale. Each value is a mean from three experiments. The broken line represents the theoretical curve drawn with a slope of 58 mV/tenfold change in $[K^+]_o$.

about 25 % after 5 min (5 experiments, average 15 %). The changes of contour of the responses occurred in two stages. First, after 2 min, the early depolarization was lowered, the plateau phase extended and the response duration prolonged. Then the rate of fall increased and the shape of the response became almost triangular. After 10 min the heart was still beating.

Generally, the modifications of the electrical activity caused by K^+-free solutions were weak and slow to appear. On the contrary, as soon as the heart was rinsed with the normal solution, rapid and marked variations always occurred; the diastolic potential increased considerably (by 24 mV after 10 sec; $n = 10$) and sometimes the membrane potential reached 100 mV. The response height increased by 20 % but the top remained under the level of the last responses recorded with the K^+-free solution. The rhythm was somewhat slowed.

Effect of calcium ions

The removal of Ca^{2+} (Fig. 2c) from the bathing solution produced an important depolarization (25 mV) which abolished the electrical activity after 60 sec. Previously, whilst the membrane was becoming depolarized, oscillations and double-peaked responses were seen. The restoration of Ca^{2+} to the bathing solution resulted in the recovering of the normal characteristics of both diastolic potential and responses.

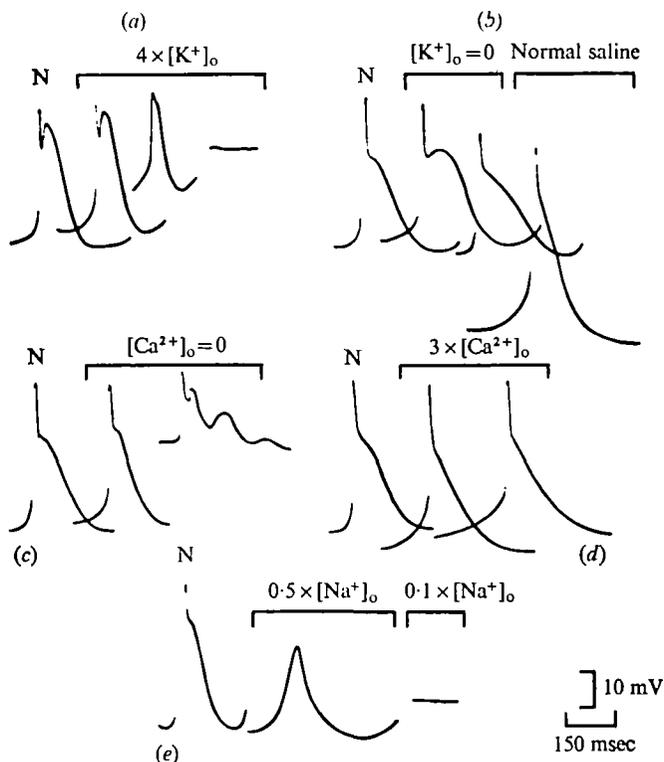The effects of excess calcium (40 mM/l, i.e. three times the normal $[Ca^{2+}]_o$) are

Fig. 2. Effect of various ions on spontaneous activity. All the tracings in the figure are drawn and the first response of each sequence represents the normal control (N). (a) Effect of a four-fold change in the $[K^+]_o$. (b) effect of K^+-free solution. (c) Effects of Ca^{2+}-free solution. (d) Effect of excess Ca^{2+}. (e) Effect of Na^+-deficient solution.

shown in Fig. 2(d). The membrane was hyperpolarized by about 6 mV and the response height was increased by the same value 30 sec after the introduction of the modified saline. Its contour was altered as follows: the rate of rise was progressively slowed down, the early rate of fall was increased but its terminal part was decreased. The increase of the diastolic potential was accompanied by a decrease in frequency which persisted somewhat after normal saline had been re-introduced.

Effect of sodium ions

Normal $[Na^+]_o$ in the bathing fluid was 306 mM/l. The study of the effect of excess sodium ions on the membrane was not easy due to the difficulty in separating the osmotic and direct membrane effects of the Na ions. Hence systematic study of such salines was not undertaken. Besides, some experiments ($n = 8$) revealed that the effect of excess Na saline were not consistent. Nevertheless we sometimes observed that a solution containing 460 mM/l of Na^+ could restore a normal contour to electrical responses whose rate of rise was particularly low.

Fig. 2(e) illustrates the effect of Na^+-deficient solutions (61 mM/l; 30·6 mM/l). The rhythm and the amplitude of the responses decreased and their contour, prin-
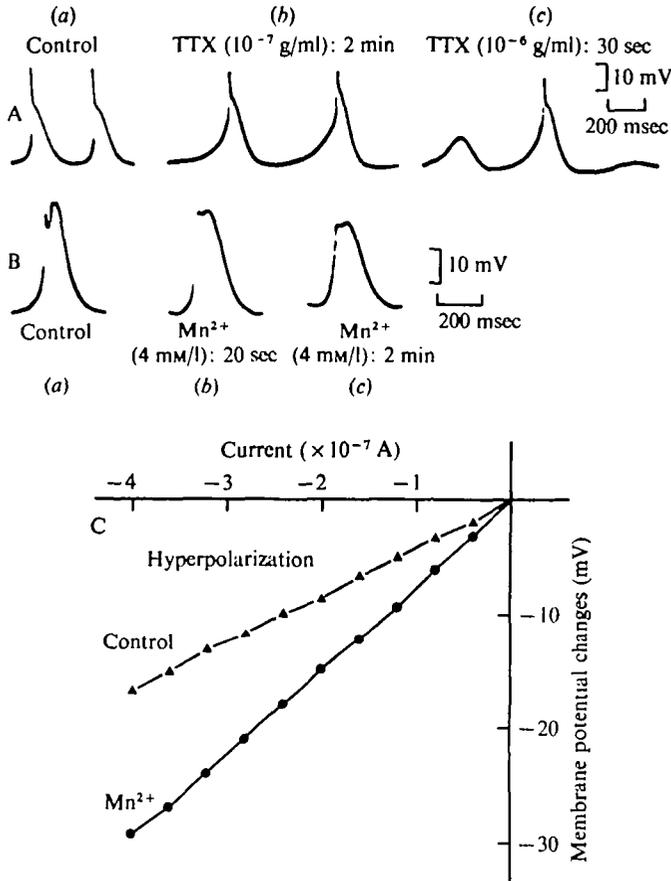
Fig. 3. (A) Effect of TTX (10^{-7} g/ml) on spontaneous activity. (a) Control; (b) TTX after 2 min; (c) TTX (10^{-6} g/ml) after 30 sec. (B) Effect of Mn^{2+} (4 mM/l) on spontaneous activity. (a) Control response with a high, humped plateau; (b) Mn^{2+} after 20 sec; (c) Mn^{2+} after 2 min. (C) Changes in the membrane resistance (R_M) measured under normal conditions (triangles) and when Mn^{2+} (4 mM/l) was added to the saline (circles).

cipally the rising phase, were slowed the most when $[Na^+]_0$ was lower. When $[Na^+]_0$ was reduced to one-tenth of the normal concentration, the membrane was depolarized and activity ceased.

Effect of tetrodotoxin and manganese ions on the spontaneous electrical responses

Effect of TTX

Fig. 3A shows the effect of TTX on the spontaneous responses. At the concentration of 10^{-7} g/ml the rate of rise was decreased (b) and the response amplitude tended to diminish, but this modification appeared more tardily than the reduction of the rate of rise. At 10^{-6} g/ml, after 30 sec, some abortive responses were recorded (c), and the activity stopped suddenly before the top of the responses was greatly lowered. It could be supposed that this effect was caused by blocking the activity of the cardiac ganglion rather than by an inactivation of the myocardial membrane.
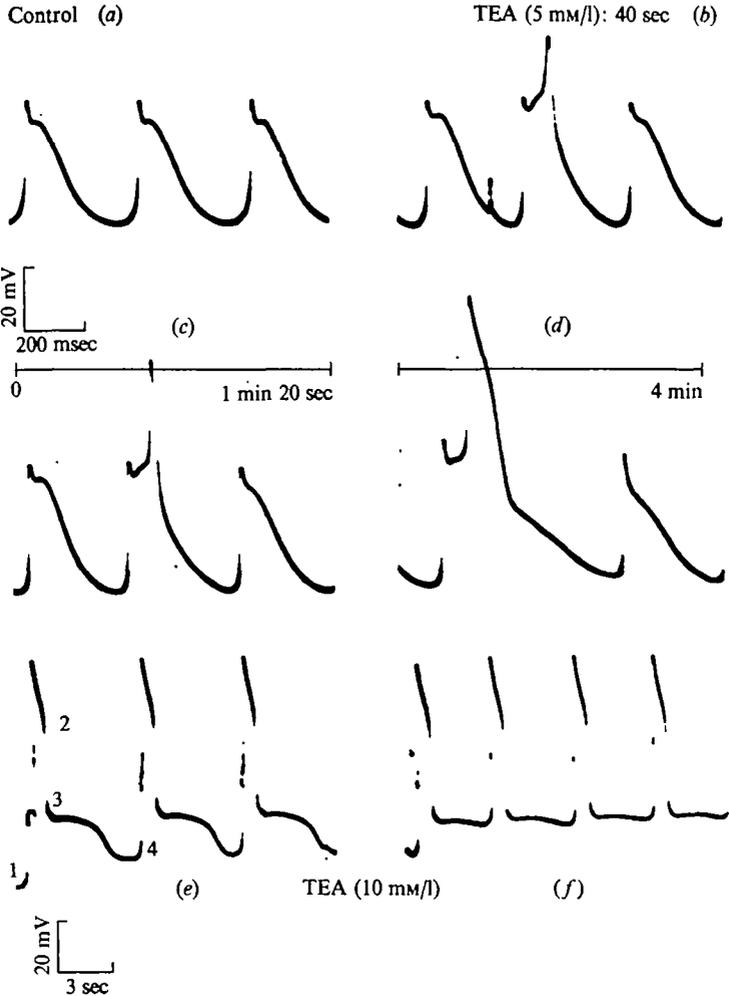
Fig. 4. Effect of TEA (5 mM/l) on the spontaneous activity. (a) Control. (b) TEA after 40 sec. (c) TEA after 1 min 20 sec. (d) TEA after 4 min. (e, f) TEA-induced activity and the different levels (1, 2, 3, 4) of the membrane potential (10 mM/l). The horizontal lines represent the zero reference level.

Effect of manganese

An example of the effect of Mn^{2+} (4 mM/l) on the electrical activity is shown in Fig. 3B. First, it lowered the plateau phase (b); this was particularly notable when this latter was humped (a). If, under 'normal' conditions, some responses exceptionally included a spike arising on the plateau, Mn^{2+} suppressed it quickly. The frequency of the heart beats and the early rate of rise were slightly increased. After 2–3 min. Mn^{2+} produced a progressive decrease of the resting polarization, and in some preparations only a regular oscillation remained. As indicated in Fig. 3(c), the membrane resistance (R_M) increased markedly.
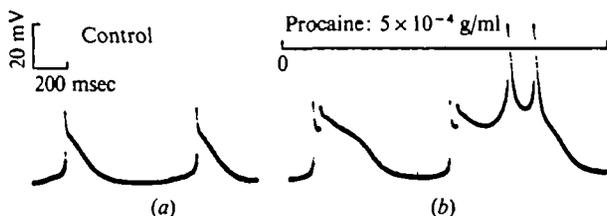
Fig. 5. Effect of procaine (5×10^{-4} g/ml) on the spontaneous activity. (a) Normal control. (b) Procaine, after 70 sec. In (b) the horizontal line represents the zero reference.

Changes in membrane electrogenesis induced by tetraethylammonium

Effect of TEA on the spontaneous activity

The heart was very sensitive to TEA, whose dominant effect was the induction of a spike superimposed on the usual responses. The detail of this effect is typically illustrated in Fig. 4 (a–d). The membrane was slightly depolarized (5 mV) and an elevation of the plateau phase occurred 45 sec after the introduction of 5 mM/l TEA. But the most striking effect was the appearance of a spike (TEA-spike) arising from the plateau of some responses (b). At the beginning this spike looked like those which appeared spontaneously at random in the normal saline. The phenomenon became progressively more pronounced and the amplitude of the whole response (TEA-response) was then 70 mV after 1 min 20 sec (c). The repolarization then included two phases of which the first was relatively rapid, the second slower. During the following minutes the spike height increased and the TEA-response could reach 85–100 mV with an overshoot of 20–25 mV. After 4 min the repolarization profile became more complex, due to the lengthening of the spike duration and the appearance of a high level plateau (d). After 7 min the activity was not stabilized, as the different rates of fall decreased progressively. The whole response lasted 650 msec to 1 sec. These TEA-responses appeared at a ratio of 1 for every 20 unmodified, spikeless responses. These latter had a regular but slower rhythmicity than that recorded under normal conditions. Those which followed a TEA-response arose in a relatively abrupt way, at the end of the slow repolarizing phase. So long as the diastolic potential had not returned to its maximum value, the level of both top and plateau were lowered. The solutions containing 10 mM/l of TEA accentuated the effects observed in 5 mM/l. The relative frequency of TEA-responses increased so that after about 3 min only these responses were recorded. Further changes then occurred as shown in Fig. 4 (e, f). It is important to describe such progressive changes during which several stable equilibrium levels of the membrane potential appeared. The part of the trace situated in the lower left corner of Fig. 4(e) indicates the value of the maximum diastolic potential with TEA (-63 mV = level 1). It can be seen that the repolarization of the TEA response was quite complex, showing several levels more or less stable. Succeeding the first plateau (level 2) the repolarization remained stable at a third level (-40 mV) before reaching a new diastolic potential (-53 mV = level 4) distinct from level 1. Fig. 4(f) shows the final state of the same preparation; the diastolic potential is now stabilized at level 3. In some cases we could observe a blocking near the zero potential (level 2) which was stable and only a few oscillations appeared. This event will be studied in detail below. With 20 mM/l of TEA the
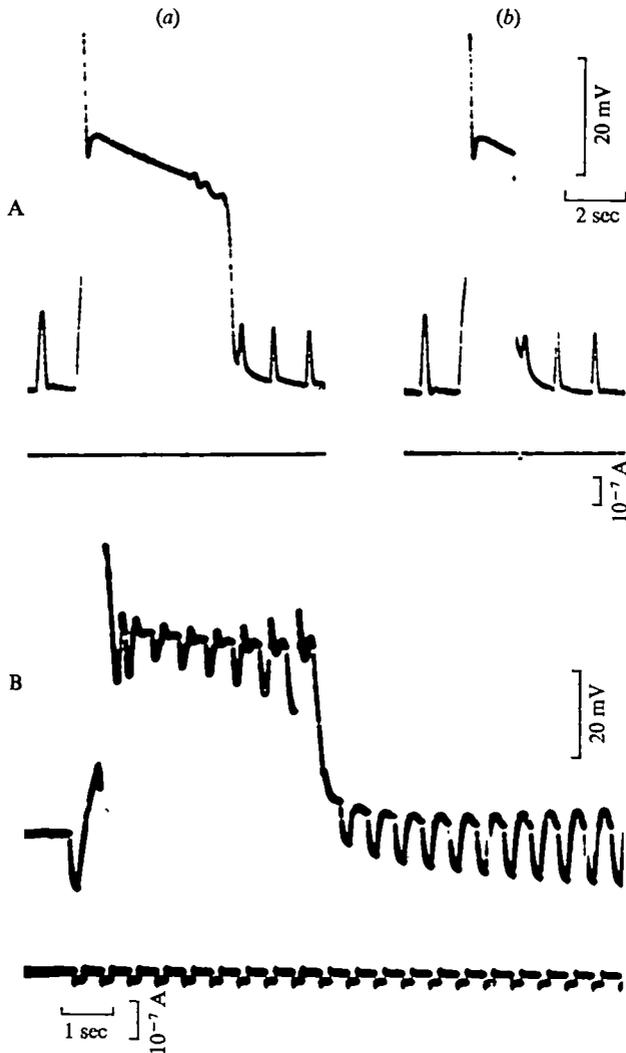
Fig. 6. Responses including a long-sustained plateau (a) induced in TEA-solution (10 mM/l). (A) A brief inward pulse repolarized the membrane in an all-or-none manner (b). (B) Changes in R_M. Detailed explanation is in text.

behaviour of the myocardium was the same but the ultimate depolarization of the membrane was greater (about 35 mV after 4 min).

The sensitivity of the heart to TEA was still displayed by poor preparations for, when introduced, it stimulated the heart and transformed abortive responses into large responses.

Other substances, such as caffeine and procaine, induced responses with spikes. Procaine (5×10^{-4} g/ml) did not appreciably modify the resting membrane potential (Fig. 5). The response plateau was markedly prolonged and the frequency slightly decreased. The activity was characterized by double or multi-peaked responses 60–80 sec later (b). The spikes were elicited at the end of the plateau phase and the total amplitude reached 70–80 mV. Caffeine (10^{-3} g/ml) increased the spontaneou(

A

(a)   Control   (b) TEA (10 mM/l): 20 sec

(c)   TEA: 30 sec   (d) TEA: 60 sec] 20 mV

] 2·5 × 10⁻⁷ A

250 msec

Current (× 10⁻⁷ A)

−3   −2   −1

B

Hyperpolarization

Control

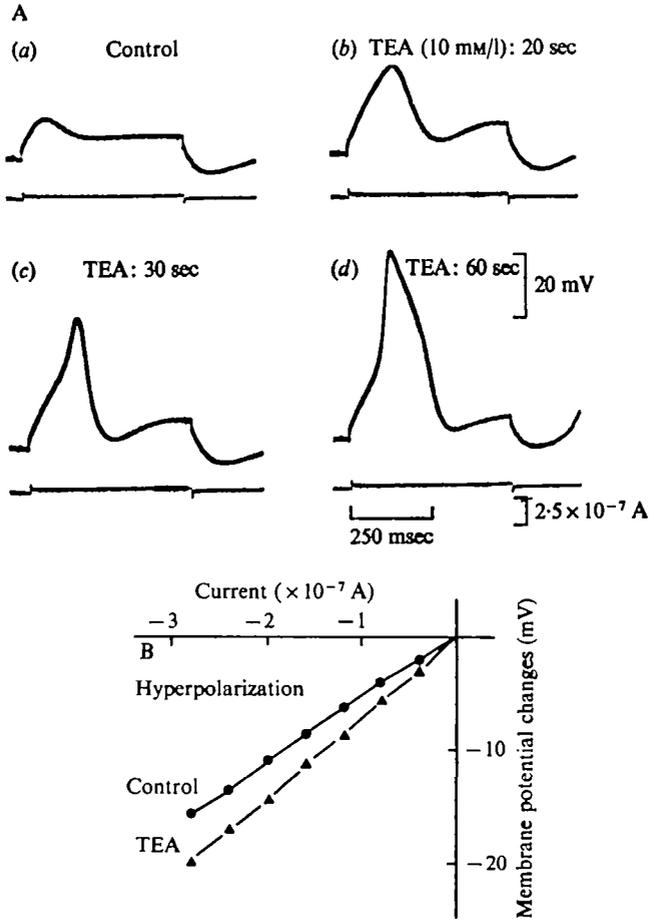TEA

−10

−20

Membrane potential changes (mV)

Fig. 7. (A) TEA-induced activity. (a) Response of the resting membrane to a constant outward pulse in the normal saline. (b–d) When TEA was added in the saline a TEA-spike was triggered. (B) Changes in R_M in TEA solution (triangles) with respect to that recorded in normal Ringer (circles).

rhythmicity. A spike arose from the plateau of each response; its height increased progressively and after 4 min the responses overshot by 10 mV. All the experiments showed good reversibility when the spike-inducing substance was withdrawn from the bathing solution.

Some pharmacological substances like acetylcholine or DDT, added in TEA-solution, often induced a more frequent blocking of the membrane potential near the electrical zero (level 2). Moreover, this property remained long after the heart was rinsed with normal saline containing TEA. We took advantage of this effect to study membrane properties, namely the changes in resistance, during the sustained plateau at level 2. Fig. 6A (a) shows a long-lasting spontaneous TEA-response (5·5 sec). Longer durations (15 sec) could be observed in the same experiment. It can be seen in Fig. 6A (b) that a brief inward current pulse of sufficient intensity was able to repolarize the membrane in an all-or-none manner. Fig. 6B illustrates the changes in the membrane resistance during a spontaneous TEA-response. The transition
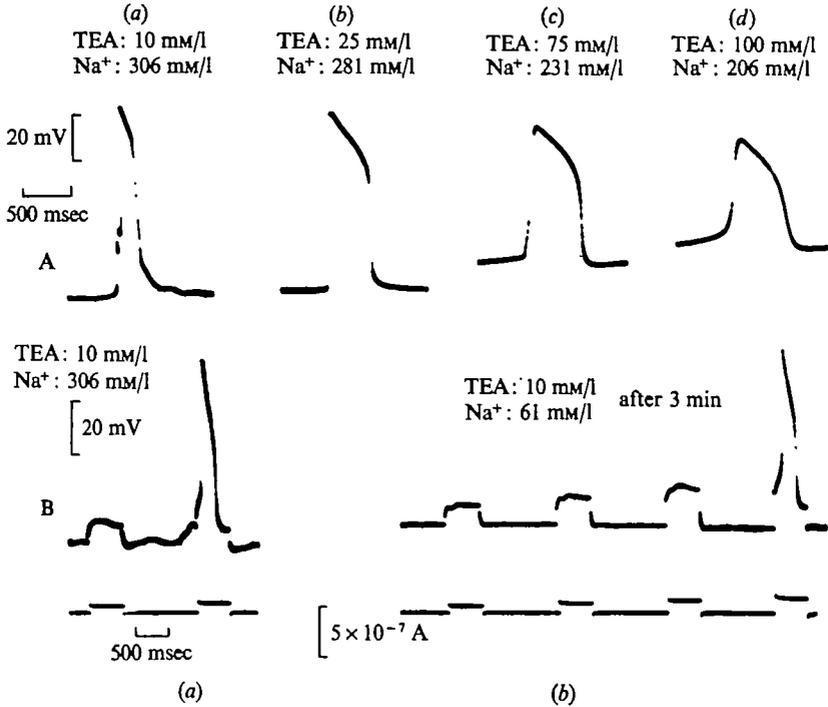
40

Fig. 8. Effect of Na⁺-deficient solutions on the TEA-induced activity. A: (a) control; (b–d) Na⁺ was replaced by TEA in steps. B: (a) TEA-induced activity; (b) effect of increments of depolarizing pulses when $[Na^+]_o$ was reduced to one-fifth.

from the diastolic potential to the long sustained plateau phase and the change in the opposite direction were accompanied by a loss of R_M (approximately 50%), and when the plateau phase was terminated R_M increased until it returned to its resting value.

Membrane excitability in a TEA medium

The occurrence of large responses to TEA led to the inference that membrane properties controlling excitability were modified. This was tested by applying constant depolarizing current pulses to the heart at rest in 10 mM/l TEA (Fig. 7). In A, the control (a) displays a weak active response. A few seconds after the introduction of TEA into the bathing solution the membrane potential was decreased, as previously described, and the local response was enhanced (b). When this local response reached a critical value the membrane became able to generate an action potential (TEA-AP), whose amplitude was enlarged progressively (c, d). After 60–90 sec the response was stable. The membrane responded in an all-or-none manner as soon as the intensity of the depolarizing current reached a threshold value (10^{-8} A). Then progressive increments of current reduced the latency of the TEA-AP and increased its rate of rise. In the experiment illustrated in Fig. 7 the TEA-AP amplitude was 60 mV and its duration 300 msec. Its contour included several phases: a relatively rapid depolarization (1 V/sec), arising from the local active membrane response, an early repolarization corresponding to an inclined plateau, followed by a repolarization with

a higher rate of fall. Generally, just after these different phases, the membrane voltage tended to decrease again until the cessation of the anodic pulse, after which a transient hyperpolarization of the membrane occurred. Increasing the frequency of the intracellular electrical stimulation produced a reduction in height. The value of the absolute refractory period was about 1 sec.

Graph B in Fig. 7 shows that, in this experiment, TEA caused the resting membrane resistance to increase by approximately 25 %

Effect of sodium and TTX on the TEA-induced activity

Fig. 8A shows the changes in the spontaneous TEA-reponse when 25, 50, 100 mM/l Na$^+$ were replaced in steps by equimolar amounts of TEA. The records were taken from the same heart throughout the experiment. The sequence of tracings indicate that the control TEA-response (*a*) underwent modifications which concerned its profile and its time course (*b*, *c*, *d*). Both rate of rise and rate of fall were depressed and the response duration was increased to 800 msec. The membrane was gradually depolarized by 18 mV in (*d*) and the top of the response was lowered with the result that the amplitude decreased by 40 mV, i.e. one half of the control. Thus Na ions seem to be involved as charge carriers and TEA ions fail to replace them.

The substitution of 153, 245, 275 mM/l Na$^+$ by equimolar amounts of choline chloride, in the presence of 10 mM/l TEA, did not produce a consistent effect with every preparation. However, after 24 experiments several facts emerged; the Na$^+$-deficient solutions reduced myocardium excitability but this reduction did not seem to be only a consequence of the decrease of the membrane resistance. Withdrawing 90 % of the Na$^+$ always quickly suppressed TEA-AP, whatever the intensity of current might be, and furthermore all measurable local response was also abolished. When [Na$^+$]$_0$ was reduced by less than 90 %, the TEA-induced activity remained sometimes as all-or-none, but graded responses could be also recorded. The responses changed with time although [Na$^+$]$_0$ remained constant. Fig. 8B illustrates the effect of a solution in which 80 % of the Na$^+$ was substituted. In control (*a*), the first current step was below the threshold level, but the next one, slightly more intense, elicited a TEA-AP. In part (*b*), 3 min after the Na$^+$ substitution, the membrane was depolarized by a few mV and there was loss of excitability. Indeed stronger pulses were then necessary to trigger a TEA-AP whose amplitude was, moreover, less than that recorded with normal [Na$^+$]$_0$. During several minutes the triggering level continued to increase, then reached a steady state.

When TTX (10^{-6} g/ml) was added to the bathing solution containing normal [Na$^+$]$_0$ and TEA, the records showed that neither the time course nor the amplitude of the TEA-AP were significantly altered after 4 min.

Effect of calcium and manganese on the TEA-induced activity

Fig. 9A shows the effect of reducing [Ca^{2+}]$_0$ to 10 % on the control TEA-AP, whose amplitude was 62 mV (*a*). Within a few seconds the membrane was depolarized, the spike height decreased and some oscillations only remained (*b*, *c*). Increments of depolarizing pulses could no longer elicit a TEA-AP. In Ca^{2+}-free solutions to which EDTA was added the preceding results were accentuated.

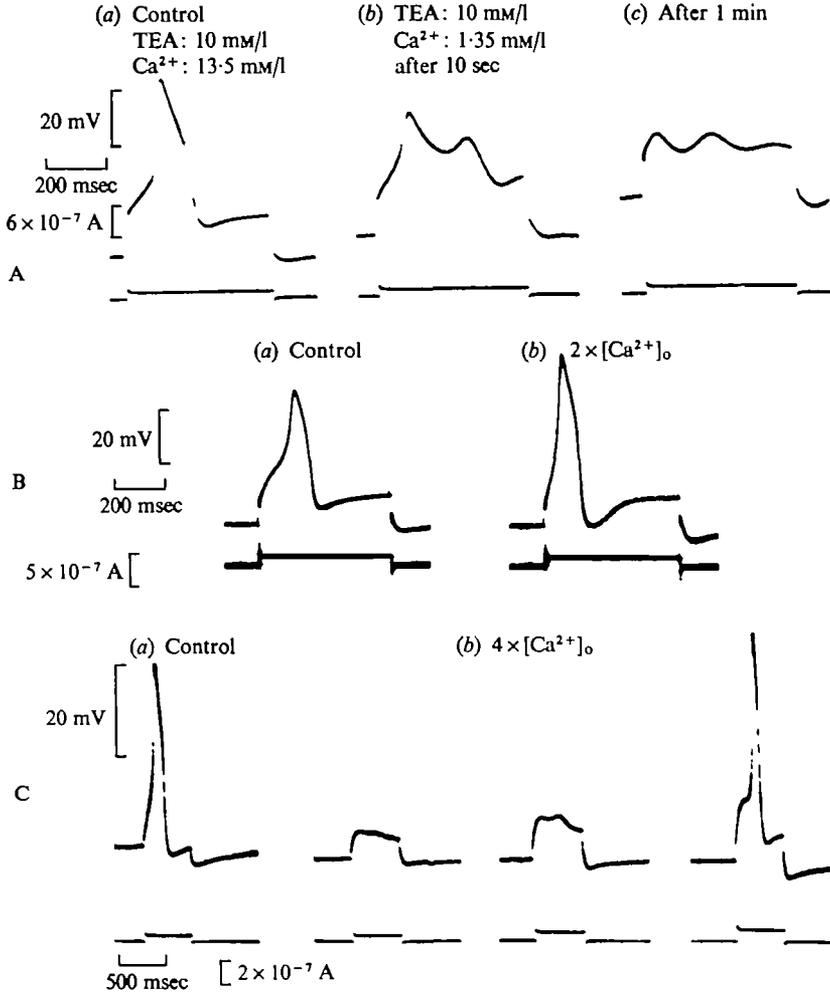Fig. 9B shows the effect of TEA in excess-Ca^{2+} solution (twice normal [Ca^{2+}]$_0$)

Fig. 9. Effect of various concentrations of Ca^{2+} on the TEA-spike. A: (a) Control; (b, c) $[Ca^{2+}]_o$ reduced to a tenth of the normal concentration. B: (a) control; (b) effect of excess Ca^{2+} (twice of normal $[Ca^{2+}]_o$). C: (a) control; (b) increments of outward pulses (four times the normal $[Ca^{2+}]_o$).

when constant anodic pulses were applied to the myocardium. The TEA-AP amplitude became greater by 10 mV while the membrane was slightly hyperpolarized. Moreover the rate of rise and membrane resistance were increased. Fig. 9C illustrates the effect of 54 mM/l Ca^{2+} on the TEA-induced activity. This fourfold change in $[Ca^{2+}]_o$ led to an increase of the response height and to a loss of excitability as shown in tracings (b). When $[Ca^{2+}]_o$ was increased by tenfold, TEA-AP could not be elicited over the ranges of the increments of depolarizing pulses tested. Briefly, optimal excess-Ca^{2+} solutions were required for the TEA-induced effect. The failure to elicit response beyond 54 mM/l Ca^{2+} prevented us from establishing an accurate relationship between response amplitude and log $[Ca^{2+}]_o$.

As shown in Fig. 10A, the TEA-AP was suppressed within 30 sec when Mn^{2+} (4 mM/l) was added to the bathing medium. Increments of depolarizing pulses could
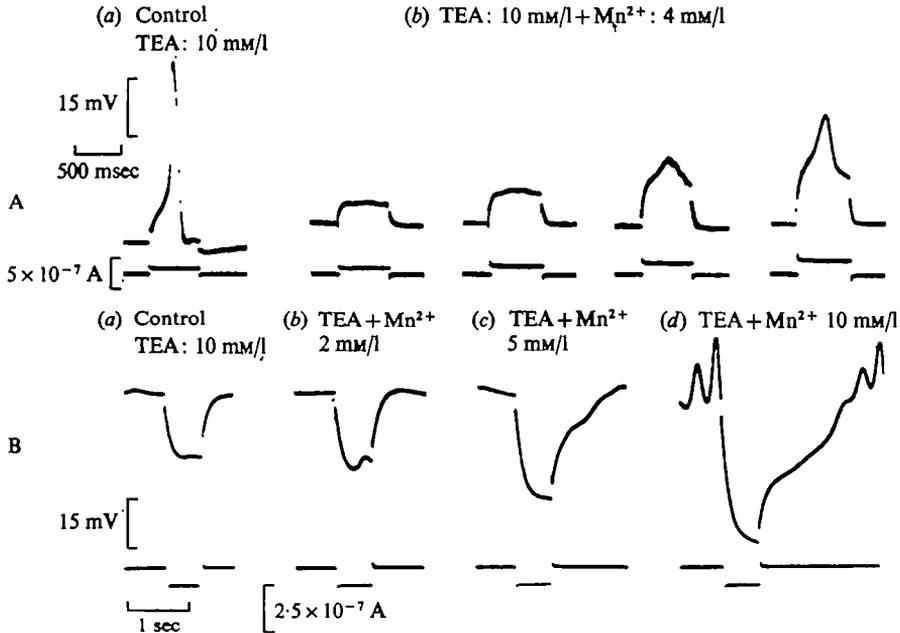
Fig. 10. Effect of Mn²⁺ (4 mM/l) on the TEA-induced activity. A: (a) control; (b) increments of outward pulses when Mn²⁺ was added in the saline. B: Evolution of R_M. (a) Control; (b–d) increase of $[Mn^{2+}]_0$.

no longer trigger a TEA-AP, and only a weak graded response was then observed (series *b*). The tracings in Fig. 10B were recorded in sequence from another cardiac fibre. They illustrate the changes in the membrane resistance when increasing concentrations of Mn^{2+} were introduced. As previously observed under normal Ringer conditions, Mn^{2+} strongly increased the membrane resistance. At the cessation of the inward pulse the membrane potential slowly returned to its resting value. It is of interest to note that in 10 mM/l Mn^{2+}-TEA solution oscillation (amplitude 10–15 mV) of the membrane potential could appear (*d*). It seemed that a relatively high amount of Mn^{2+} was required to produce such an activity because if $[Mn^{2+}]_0$ was decreased by 5 mM, this latter failed to appear.

Effect of γ-aminobutyric acid (GABA) on the TEA-induced activity

As described previously (Holley & Delaleu, 1972), GABA inhibits spontaneous normal responses of the wood-louse heart and causes the membrane resistance to decrease. The question is whether such an effect on the membrane resistance could not block the generation mechanism involved in the all-or-none activity. The effect of GABA (10^{-6} g/ml) on the TEA-AP (Fig. 11) was rapid, for within 15 sec the response was abolished and increments of depolarizing pulses could not restore the TEA-induced activity. GABA did decrease the membrane resistance. After GABA was removed from the bathing medium, anodic pulses could again trigger TEA-AP.
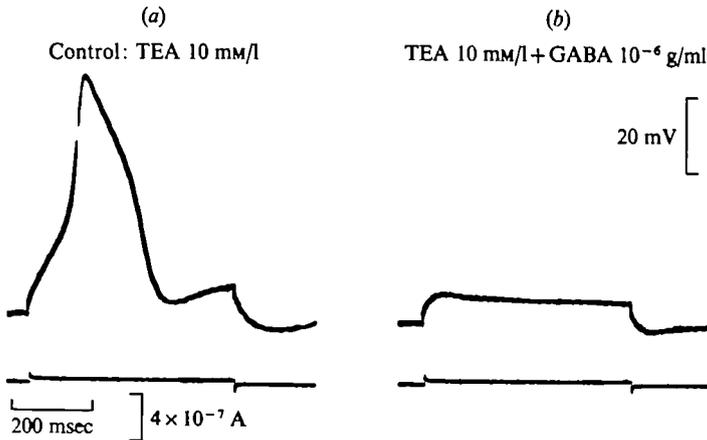
(a)
Control: TEA 10 mM/l

(b)
TEA 10 mM/l+GABA 10^{-6} g/ml

20 mV

200 msec 4×10^{-7} A

Fig. 11. Effect of GABA on the TEA-induced activity (constant pulse). (a) Control. (b) GABA (10^{-6} g/ml) after 1 min.

(a)

(c)
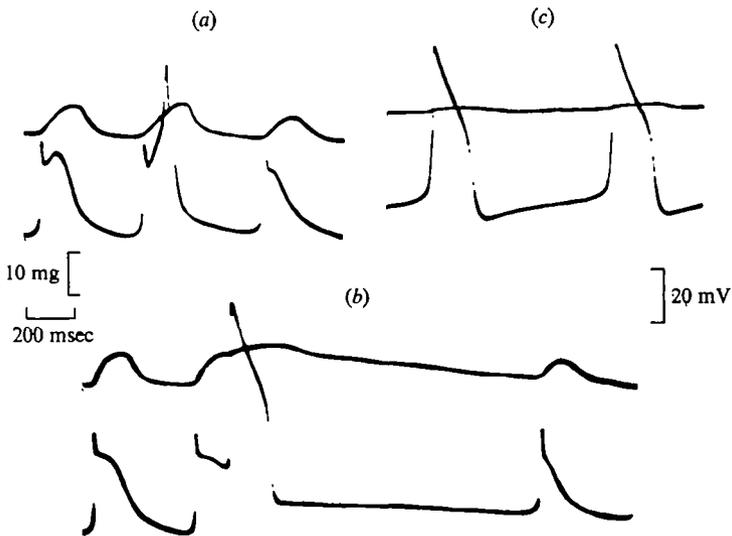
10 mg

200 msec

(b)

20 mV

Fig. 12. Excitation–contraction relationship in TEA solution (spontaneous activity). (a) TEA (10 mM/l) after 30 sec; (b) TEA after 1 min 30 sec; (c) TEA after 3 min.

Relationship between TEA-induced activity and contraction

Preceding studies had demonstrated (Holley & Delaleu, 1972) that the degree of mechanical tension and relaxation of heart fibres closely depends on the value of the membrane voltage. We attempted to investigate further the relationship between membrane potential and contraction taking advantage of the strongly modified activity during TEA treatment (Fig. 12). As shown in (a), a contraction induced by a TEA-response with a brief spike was hardly greater than that corresponding to a usual response with a humped plateau. In (b), after the spike, the membrane voltage failed to return to the maximum diastolic value and the membrane remained steadily depolarized by 10 mV. At the same time the degree of tension, although slowly

decreasing, did not fall below the maximum recorded during the usual response preceding the spike. It can be seen that the fully developed spike hardly contributed to the mechanical tension. In a further stage (c) the membrane became depolarized by about 15 mV and only TEA-spikes occurred. As a consequence of the diastolic depolarization the fibres displayed a sustained contracture, and in spite of 55 mV responses the corresponding contractions were hardly visible on the tracing. Thus it can be concluded that membrane potential variations which are the most effective in controlling mechanical tension, are those occurring near the normal resting membrane potential.

DISCUSSION

The resting membrane potential

Potassium seems to be largely responsible for the maintenance of the membrane potential. However, in *Porcellio*, this ion does not play as exclusive a role as in *Carcinus* heart studied by Lassalle & Guilbault (1970), who found that the resting potential behaved as though it was maintained by a K^+ battery. The effect of K^+-free solution and the striking consequence of re-introducing normal $[K^+]_o$ in the bathing medium which was previously deprived of potassium are worth a closer examination. It must be recalled that the hyperpolarization expected in K^+-free medium was found to be small, lasting a short time and often failing to occur at all. Anyhow, the membrane then became depolarized. This paradoxical depolarization may be interpreted as an effect of a greatly reduced potassium permeability in absence of external K^+. Hence, when the normal $[K^+]_o$ was re-introduced, the potassium permeability would be rapidly increased at a time when $[K^+]_o$ was still low, resulting in hyperpolarization. However, the hyperpolarization was observed only if the heart had been previously K^+-deprived for a long time (several minutes). This suggests another tentative explanation implying the function of a metabolic pump. Supposing that a Na^+–K^+ pump contributes to the membrane potential, the omission of K^+ would inhibit the pump, leading to depolarization and to the accumulation of Na^+ inside the cells. When K^+ was re-introduced the pump was able to function again and, what is more, was stimulated by the high $[Na^+]_i$. The hyperpolarization was caused by the efflux of the Na^+ ions which were uncoupled with the inward movement of K^+. Such a mechanism has been postulated by Meunier & Tauc (1971) to account for the behaviour of neurons in *Aplysia* in K^+-free solution. To substantiate the hypothesis of a direct role of a Na^+–K^+ pump in the maintenance of the membrane potential, further investigations making use of agents known to act on the pumping mechanism would be necessary.

Sodium contributed to some extent to the membrane potential, for it was shown that decreasing $[Na^+]_o$ slightly hyperpolarized the membrane at least as long as the activity was maintained. It may be concluded that the relative sodium permeability is of some importance at rest. Thus the heart of *Porcellio* differs from that of *Carcinus* in which the cardiac membrane potential was not altered in Na^+-deficient solutions (Lassalle & Guilbault, 1970).

The resting membrane potential has been shown to be strongly dependent on $[Ca^{2+}]_o$ as Ca^{2+}-deficient solutions caused a significant depolarization of the membrane and excess Ca^{2+} produced hyperpolarization. However, the data are not consistent

with the concept of a Ca^{2+}-battery directly controlling the membrane potential. It can be supposed that the efficiency of Ca^{2+} in determining the membrane potential depends on its ability to regulate other specific conductances. Such an interpretation was proposed by Ito, Kuriyama & Tashiro (1970), who found results rather similar to ours in the somatic muscle of the earthworm. Previously, Koketsu, Nishi & Soeda (1963), who studied nerve fibres, suggested that Ca^{2+} plays a role as a controller of Na^+ and K^+ permeabilities.

Nature of the electrical response

In a preceding paper (Holley & Delaleu, 1972) we have interpreted the spontaneous response as composed of an initial depolarizing phase of a mainly synaptic nature, followed by a weak active response of the myocardial membrane, resulting in a plateau. Is this interpretation consistent with the effects of modified ion solution and the action of permeability inhibitors? At first sight the reduction of both amplitude and rate of rise of the response in Na^+-deficient solution, as well as the blocking effect of TTX, could be regarded as supporting the view that depolarization implies the activation of a specific Na^+ permeability. However, with regard to the action of TTX, it must be kept in mind that the heart stopped before a significant reduction in response height could take place. Probably TTX acted principally on the heart ganglion, first slowing its rhythm, then blocking it. Some abortive responses, as seen in Fig. 3A (c), may be interpreted as local, myogenic responses without nerve impulses. As for the effects of reducing $[Na^+]_0$, they are not inconsistent with the junctional nature of the upstroke. Indeed the replacement of Na^+ ions by choline, thus highly reducing the amount of positive charge carriers, could result in diminishing the intensity and the kinetics of the current which generates a junction potential across chemically excitable membrane areas. However, the effects of an Na^+-deficient solution or of TTX are not conclusive enough to explain the nature of the assumed composite response. From the action of the Ca^{2+}-deficient solution it follows that very small responses (less than 10 mV) may maintain a relatively high rate of rise as long as the nerve conduction and the junction transmission are not impaired. The effect of Ca^{2+}-deficiency thus differs from that of Na^+-deficiency. The maintenance of a quasi-normal rate of rise in spite of depolarization may mean that Ca^{2+}, which does stabilize the resting membrane potential, does not play so significant a role in the rising phase of the response. As for the height reduction, it could be explained as being mainly a consequence of depolarization. It will be noted that the omission of Ca^{2+} did not markedly modify the total amount of the positive charge carriers. Therefore, if we assume that the depolarizing phase could be a junction potential, it could then be understood why the absence of Ca^{2+} ions did not considerably alter the rate of rise. This argument would not be cogent if the depolarizing phase was due to a selective increase in Ca^{2+} permeability. In this case an important reduction of the Ca^{2+} battery would result in reducing the rate of rise as well as lowering the top of the response. This was not observed. In addition it will be noted that increasing $[Ca^{2+}]_0$ did not result in raising the level of the top either. As for the slight increase in the response amplitude, it could be caused by hyperpolarization. It is difficult to demonstrate a specific intervention of Ca^{2+} and Na^+ ions in generating the plateau phase since they primarily acted on resting membrane potential and on the de-

polarizing phase. When observed, the alteration of the plateau could be only a secondary effect of the ionic modifications. The changes recorded in Mn^{2+} Ringer are more easily interpretable, for the lowering of the plateau occurred before any other change in potential and in spite of an increase in membrane resistance. Mn^{2+} is known to suppress the Ca^{2+} spike in crustacean muscle (Hagiwara & Nakajima, 1966) and to reduce the initial spike in the heart of the crayfish *Procambarus clarkii* (van der Kloot, 1970) and of the crab *Carcinus maenas* (Lassalle, 1971). Moreover, it has been shown that Mn^{2+} inhibits a 'slow channel' for both calcium and sodium which is responsible for the late depolarization and for the plateau in the vertebrate heart (Rougier *et al.* 1969; Ochi, 1970). In the light of these data, the lowering of the plateau in *Porcellio* heart can be interpreted as a result of the inhibition, by Mn^{2+}, of an active electrogenic mechanism where either Ca^{2+} only or Ca^{2+} and Na^+ were involved. This tentative explanation will be further discussed in connection with the effects of TEA.

Nature of the TEA-induced activity

TEA ions transformed the weak spontaneous response into a large spike-type response. In addition, TEA permitted outward current, when applied to the resting myocardium, to trigger large all-or-none responses. To a lesser degree, procaine and caffeine induced a similar transformation of the spontaneous response. At first sight these results can be compared, without consideration to the ionic mechanisms involved, with those observed by Fatt & Katz (1953) in crustacean muscle fibres.

The modalities of the spike appearance among spontaneous responses call for several comments. First, it will be noted that the upstroke of normal responses was only weakly modified. Its slight increase can be explained by the increase in membrane resistance. The foremost effect of TEA was to raise the level of the plateau. The spike always appeared on this plateau and never as an extension of the initial depolarization. The transformation of electrogenesis induced by TEA thus seems to concern specifically the late phase of the response. The dual nature of the normal response has been suggested previously. It is worth noting that TEA regenerative activity does arise from the very component we thought of as being active. In addition, it can be seen in Fig. 4(*b, c, d*) that the occurrence of a spike during a response resulted in a lowering of the plateau of the following, spikeless responses. This could be explained as due to a long refractoriness of the active electrogenic process. The dependence of the plateau of a given response on the earlier spikes seems an argument in support of the active nature of the plateau electrogenesis. Indeed, if the plateau was purely synaptic and strictly dependent on ganglion discharge it would be difficult to account for its modifications in consequence of the preceding, active process which specifically involved the electrically excitable areas of the myocardial membrane. As seen in various stages illustrated in Fig. 7, the TEA activity resulted apparently from an amplification of the weak normal, local response. Therefore we may make the hypothesis that the processes leading to TEA-spike electrogenesis are not fundamentally distinct in nature from those controlling the second phase of the normal response.

The question which arises now is relevant to the mechanisms implied in the modified electrogenesis. It has been shown that membrane resistance was markedly

increased in TEA solutions. It may be explained taking into account the well known property of TEA to decrease the potassium conductance (Tasaki & Hagiwara, 1957; Wermann & Grundfest, 1961). We may suppose that, in normal Ringer, the synaptic or experimental depolarization failed to generate an all-or-none action potential because the outward ionic flux, mainly of potassium, prevented the inward ionic positive current to reach a triggering level. On the other hand, when TEA was present, the critical level for the spike-generating specific conductance could be reached as a consequence of a decrease in potassium conductance. Currents other than the potassium current may contribute in limiting the spike-generating mechanisms. The spike-suppressing effect of GABA in TEA was not probably due to a specific change in K^+ permeability, for other ionic conductances such as that of chloride ions were shown to be increased by GABA in crustacea (Ochi, 1969).

The repolarization of the TEA-AP shows a complex profile where several critical levels can be discerned. At first, it can be seen that the response includes a plateau which, under some circumstances, can last longer. These TEA-spikes resemble the normal action potentials in mammalian myocardium (Coraboeuf & Weidmann, 1949), the TEA-spikes observed in the squid giant axon (Tasaki & Hagiwara, 1957) and in the crustacean skeletal muscle (Fatt & Katz, 1953). Since the value of the total resistance, which is at first below that of diastolic resistance, increases progressively during the long plateau, it could be conceived that the mechanisms which generate and maintain these responses are slowly inactivated during this time. Generally this long plateau ends abruptly as shown in Fig. 6A (a). Thus one can consider that the plateau reveals a new stable state of the membrane. Moreover, if an inward current pulse with sufficient strength is applied during the plateau, the polarity reverses in an all-or-none manner. The existence of a threshold for the repolarization, as for the depolarization, supports the view that the process of initiation and of abolition of the TEA-AP represents a transition of the membrane between two stable levels. Under normal conditions, i.e. when no step of current is applied during the plateau, the repolarization occurs abruptly when the slow increase in membrane potential reaches a critical threshold for abolition. Thus, in presence of TEA, the myocardial membrane of *Porcellio* behaves similarly to that of the crustacean skeletal muscle membrane (Fatt & Katz, 1953), the vertebrate myocardium (Weidmann, 1951, Garnier *et al.* 1969) and the squid axon (Tasaki & Hagiwara, 1957). However, the phenomena which have just been discussed concern a late phase of the effect of TEA on the preparation. As can be seen, an additional complexity appears when the early stages of this action are considered. The maximum level of polarization described previously is not the maximum diastolic potential observed in TEA. As seen in Fig. 4(e, f), it is clear that this level is not established after a slow change in the maximum diastolic polarization but really by a lengthening and a stabilization of the lower plateau (level 3) in the first complex responses (Fig. 4c, d). Previously we have made a distinction between a level 1 and level 4. However, it is not certain that this distinction is relevant and we shall not discuss it at any greater length. However, it actually seems that the distinction of only two stable levels is not enough to describe the membrane behaviour in TEA-solution. We cannot interpret the significance of the presence of these three levels, but it is necessary to point out that the structure of

ur preparation is more complex than that of the giant axon. Indeed, the presence of several classes of membrane areas, namely synaptic and non-synaptic, contributes to this complexity.

The question which arises is to define the nature of the ions involved in the TEA-induced activity. First of all, it seems established that the TEA-AP is not due to mechanisms requiring only one kind of ion and one may assume that both Ca^{2+} and Na^+ are involved in one way or another. However, it must be pointed out that the changes in the thresholds and the dependence of the resting membrane potential on Ca^{2+} and, to a less degree, on Na^+ makes the interpretation difficult.

The experiments during which TEA was partly substituted for Na^+ supported the view that, unlike the skeletal muscle fibre of Crustacea (Fatt & Katz, 1953), the heart membrane of *Porcellio dilatatus* requires the presence of Na^+ ions so as to develop a spike-type activity. In addition, we suppose that TEA does not have an important charge-carrier function. Such a conclusion may be also implied from the experiments during which a constant concentration of TEA was added to the Na^+-deficient solution. However, as mentioned above, in 24 experiments we failed to establish a firm relationship between the action potential amplitude and log $[Na^+]_0$. Although the role of Na^+ is important, its exact nature remains difficult to define.

The role of Ca^{2+} is no more easily understood although the TEA-AP behaves at first sight like the Ca^{2+} spikes of various tissues when $[Ca^{2+}]_0$ is modified; for example, in the lowering of the top of the upstroke in Ca^{2+}-free solutions, and the enhancement in excess Ca^{2+} (Hagiwara, 1966; McCann, 1971). However, the detailed interpretation is made difficult by the variations in resting membrane resistance and potential occurring in such modified solutions. The suppression of the spike in one-tenth of the normal $[Ca^{2+}]_0$ may be a consequence of a large depolarization. The enhancement of the TEA-AP in excess Ca^{2+} may be partly attributed to the increase in the membrane resistance. Just as for Na^+, large individual variations of the relationship between response height and log $[Ca^{2+}]_0$ did not allow us to define the actual role of the Ca^{2+} battery in the measured changes.

It may be concluded that the TEA-AP is neither a pure Na^+-spike nor a pure Ca^{2+}-spike. The relative involvement of both ions cannot be stated at this time. The ineffectiveness of TTX in altering the TEA-AP seems surprising if the TEA-AP is not a pure Ca^{2+} spike. However, a tentative explanation can be given if we refer to Rougier *et al.* (1969), who reported that in the vertebrate myocardium TTX acts selectively on the 'rapid' Na^+ inward current but does not modify the sodium component of the 'slow' inward current, to which the Ca^{2+} ions also contributes. The suppressing action of Mn^{2+} ions on the TEA-AP does not imply necessarily that Ca^{2+} ions are only involved. Indeed, if Mn^{2+} ions do suppress pure Ca^{2+} spikes (Fatt & Ginsborg 1958; Hagiwara & Nakajima, 1966; McCann, 1971), they also inhibit the calcium–sodium 'slow' channel in the myocardial membrane of vertebrates. Thus, in *Porcellio dilatatus* myocardium the electrogenic mechanisms involved in the TEA-induced activity exhibit some common features with the 'slow channel' in the vertebrate myocardium membrane. As for the oscillation of the membrane potential, recorded when Mn^{2+} was added to the bathing medium containing TEA, a tentative explanation could be that Mn^{2+} itself contributed to this activity. This ypothesis is borrowed from Ochi's studies (1970) on the guinea-pig heart in which a

slow inward current was observed when Mn^{2+} was added to a solution lacking both Ca^{2+} and Na^+.

As for TEA, both caffeine and procaine elicited spikes arising from the response plateau. We are not in a position to explain them at the present time owing to the fact that we only studied in detail the TEA-AP. However, it is interesting to note that in both cases the spike appeared solely when the plateau had been previously elevated as a consequence of the action of the chemicals. Moreover the spike never prolonged the initial upstroke, which we have already interpreted as a junctional potential. Thus the change in electrogenesis induced by caffeine and procaine resembles the action of TEA. This supports the view that, like TEA, they act uniquely on the weak active component of the composite response and thus confirm the dual nature of the usual response. As for the actual mechanism underlying the activation of the active component we can only refer to Chiarandini, Reuben & Brandt (1970), who reported that the caffeine-spike was caused by an increase in the Ca^{2+} influx in crayfish muscle and to Ozeki, Freeman & Grundfest (1966), who interpreted the procaine-spike in the same preparation as being due to a change in the membrane permeability to divalent ions.

CONCLUSION

The important fact which emerges from the different results reported above and in the previous paper is that the later component of the electrical response of the heart of *Porcellio* is liable, under some experimental circumstances, to enlarge and thus to present salient features, very similar to those of membranes displaying an all-or-none regenerative activity. Since in some cases large spikes could be recorded under apparently normal conditions, the question which arises is whether this activity has any functional significance. Firstly, it can be noted that the usual responses without spikes are perfectly able to produce a powerful contraction of the heart tube. In addition, as described above, the spike-type large reponses induced by TEA only bring a relatively small addition to the contractile power. Would the spike-type activity play a part, by ameliorating the synchronization of the activation, in the whole heart? It must be pointed out that, owing to the physiologically syncytial character of the myocardium and to its small size in comparison with the space constant, a motor nerve input distributed to a relatively low number of neuromuscular junctions can be sufficient to assure the homogeneous electrical activation of the heart. As a matter of fact it was always noted that the heart beat in a synchronous manner when the responses displayed no spike. But in addition we have also established that the level of depolarization and the duration of the later component of the response, in particular its plateau, controlled the strength of the contraction. Thus it is possible to consider the mechanisms of specific conductances, responsible for the plateau, as means of regulating the power of the cardiac pump. Under these conditions the spikes sometimes observed could correspond to a non-graded extreme response at the limit of the normal graded physiological regulatory process.

In *Limulus* the whole electrical response is considered purely junctional (Parnas, Abbott & Lang, 1969). In some Crustacea an active response is admitted, but it seems only to concern the brief initial depolarization and its physiological role is not known (van der Kloot, 1970), the sustained depolarization being apparently entirely synaptic

Brown, 1964; van der Kloot, 1970). Thus the heart of *Porcellio* provides a third example where the initial upstroke seems chiefly synaptic and where the sustained depolarization brings into play active mechanisms which are functionally effective although only moderately developed.

SUMMARY

1. The effects of various ions and chemicals were tested on the resting or active membrane of the heart of the wood-louse *Porcellio dilatatus*.

2. The curve relating the resting membrane potential to log $[K^+]_o$ was found to correspond with the theoretical curve expected from the Nernst equation at higher concentrations only. Excess K^+ decreased both amplitude and rate of rise of the response while the rate of decline was increased. In K^+-deficient solutions the duration of the plateau phase was at first increased, then depressed. The addition of K^+ to a bathing medium deprived for several minutes of this ion caused a large increase in the membrane potential and in the response height. The way in which the membrane was seen to react was tentatively attributed to an electrogenic active pumping mechanism.

3. In Na^+-deficient solutions, the rate of rise and the height of the response were reduced while the resting membrane potential was decreased.

4. Ca^{2+}-deficient solutions depolarized the membrane and decreased both amplitude and duration of the response. Cessation of activity occurred in Ca^{2+}-free solution. In excess calcium the membrane was hyperpolarized. The rhythm and the rate of rising were decreased and the plateau phase depressed.

5. TTX blocked the heart activity, probably by acting upon the heart ganglion. Mn^{2+} depressed especially the humped plateau (when present) of the spontaneous responses.

6. TEA, caffeine and procaine transformed spontaneous activity of weak amplitude into large and complex overshooting responses. In TEA solutions, several stable levels of polarization were observed. Contrary to what occurred in the normal solution, depolarizing current pulses could trigger large all-or-none action potentials when TEA was present.

7. The TEA-induced regenerative response was analysed with the help of an intracellular stimulating current when $[Na^+]_o$ and $[Ca^{2+}]_o$ were varied. Additional data were obtained by applying TTX, Mn^{2+} or GABA. From the results, both Ca^{2+} and Na^+ were thought to be involved in the ionic currents underlying spike-type activity.

8. The spike-generating effect of TEA has been attributed to its property of increasing the membrane resistance and of allowing the ionic conductances which generate the weakly active component of the normal response, the plateau, but not the initial upstroke, to be amplified regeneratively.

9. The large spikes elicited by TEA were found relatively less effective than weak sustained depolarization in inducing strong contractions.

10. The functional significance of the data was tentatively interpreted by comparison with the properties of the heart of *Limulus*, Crustacea and vertebrates.

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