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IMPORTANCE OF CALCIUM IN THE ACTIONS OF SOME DRUGS THAT STIMULATE THE ISOLATED HYPODYNAMIC FROG HEART

BY

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Eight drugs that stimulate the isolated hypodynamic frog heart have been tested for their ability to stimulate hearts freshly perfused in calcium-free Ringer solution. Ouabain and digitoxigenin regularly caused stimulation, veratridine did so occasionally. Hydrogen peroxide, tannic acid, paullinia tannin (from *Paullinia pinnata*, Linn.), sodium oleate and sodium caprylate did not stimulate. The stimulant action of ouabain could be prevented or greatly reduced by prior perfusion with either of the two tannins or with hydrogen peroxide. Hearts perfused for 3 to 4 hr with calcium-free Ringer solution, or hearts perfused for shorter periods of time with calcium-free Ringer solution containing the disodium salt of ethylenediaminetetra-acetic acid, behave unusually in that when perfusion with low-calcium Ringer solution is resumed twitch tension does not return for periods ranging from 10 to 90 min. Ouabain is unable to stimulate these "calcium-depleted" hearts. It is suggested that hydrogen peroxide, the two tannins, oleate and caprylate stimulate by causing the heart to increase the uptake of calcium from the perfusion fluid to a superficial site where calcium is necessary for the propagation of excitation from the cell membrane inwards. A special feature of the action of cardiac glycosides may be their ability to enable the heart to utilize in a similar way the intracellular stores of calcium.

Many drugs can stimulate the isolated hypodynamic frog heart. They include cardiac glycosides and their aglycones, some veratrum alkaloids (Krayer & Acheson, 1946), lipoids and sodium oleate (Clark, 1913), sodium caprylate (Elliot, Kipple & Hall, 1947), two tannins (Broadbent, 1962), hydrogen peroxide and drugs forming peroxides in their solutions (Krayer, Linstead & Todd, 1943), and calcium. The drugs studied in this paper have the following features in common. First, stimulation is clearly seen in hypodynamic frog hearts. Secondly, in normal hearts stimulation is minimal and toxic effects ensue rapidly. Thirdly, if there is sufficient calcium present in the perfusion fluid the usual outcome is systolic arrest of the ventricle. Cardiac glycosides are of outstanding therapeutic importance, and it was hoped that a comparative study of the drugs on the hypodynamic frog heart might indicate some special feature in the mode of action of cardiac glycosides.

METHODS

Approximately 200 frogs were used in these experiments. Frog hearts were dissected and perfused as previously described (Broadbent, 1962). The construction of the perfusion assembly which permits the simultaneous recording of heart movements and cardiac output

has been described by Stanbridge (1962). Perfusion fluids were fed to the heart from Marriotte bottles raised above the level of the heart and excess fluid was removed by continuous suction through a tube, inside the venous cannula, connected to a filter pump. This arrangement kept the venous pressure constant and permitted continuous change of the perfusion fluid in contact with the heart. Experiments were performed at room temperature, and the frogs (*Rana temporaria*) stored at 3° C were allowed to warm up 24 hr before use. The hearts were made hypodynamic by perfusion with low-calcium Ringer solution which had the following composition: NaCl 6.5 g (112.0 mM), $CaCl_2$ 0.03 g (0.27 mM), KCl 0.14 g (1.88 mM), $NaHCO_3$ 0.5 g (5.95 mM), and distilled water to one litre. In calcium-free Ringer solution all calcium chloride was omitted.

To study the stimulant action of the drugs in calcium-free Ringer the hearts were first perfused with low-calcium Ringer until heart rate, ventricular movements, and cardiac output were steady. Then they were perfused with calcium-free Ringer for 5 min, then with calcium-free Ringer with the addition of the drug under test for 30 min, and finally with low-calcium Ringer. In control experiments hearts were perfused with low-calcium Ringer until steady, then with calcium-free Ringer for 35 min, and finally with low-calcium Ringer. To test for antagonism between ouabain and the other cardiotonic drugs in calcium-free Ringer the hearts were perfused with low-calcium Ringer until heart rate, ventricular movements and cardiac output were steady. Then they were perfused with calcium-free Ringer for 5 min, then with calcium-free Ringer containing the drug under test usually for 10 min, then with calcium-free Ringer containing the drug under test and ouabain 2×10^{-6} usually for 30 min, and, finally, with low-calcium Ringer.

To study the action of ouabain on calcium-depleted hearts two types of experiment were employed. In one type of experiment edetic acid (ethylenediaminetetra-acetic acid) was used. Hearts were perfused with low-calcium Ringer, then with calcium-free Ringer containing edetic acid 1×10^{-4} or 5×10^{-5} for 10 min, then calcium-free Ringer containing edetic acid and ouabain for 30 min, and finally with low-calcium Ringer. In control experiments hearts were perfused with low-calcium Ringer, then with calcium-free Ringer containing edetic acid for 40 min, and finally with low-calcium Ringer. In the second type of experiment the hearts were perfused first with low-calcium Ringer, then with calcium-free Ringer for periods of from 3 to 4 hr, and then with calcium-free Ringer containing ouabain for 30 min, and finally with low-calcium Ringer. In control experiments hearts were perfused first with low-calcium Ringer, then for 3 to 4 hr with calcium-free Ringer, and finally with low-calcium Ringer.

The cardiotonic drugs and the concentrations used were as follows: digitoxigenin (National Biochemical Corporation) 2.5×10^{-5}, hydrogen peroxide (B.D.H.) 1×10^{-5}, ouabain *U.S.P.* (National Biochemical Corporation) 2×10^{-6}, paullinia tannin prepared from *Paullinia pinnata*, Linn., as described by Bowden (1962), 1×10^{-4}, sodium caprylate (B.D.H.) 1.6×10^{-4}, and veratridine (Bios Laboratories Inc.) 1×10^{-5}. Digitoxigenin was dissolved in 95% ethyl alcohol; the final concentration of alcohol in the Ringer solution was 0.5% and the same concentration of alcohol was added when appropriate to low-calcium Ringer and calcium-free Ringer solutions. In experiments employing edetic acid the disodium salt (Analar) was used.

RESULTS

Stimulant actions in calcium-free Ringer. In control experiments hearts were perfused for 35 min in calcium-free Ringer. The initial change from low-calcium Ringer to calcium-free Ringer caused the ventricular contractions to become very weak so that movements of the heart lever usually ceased, the heart rate fell and cardiac output ceased. Waves of slight stimulation sometimes occurred if the calcium-free Ringer in contact with the heart was not changed continuously and rapidly. This was probably due to calcium from the heart gradually increasing in the small volume of solution contained in the venous cannula. On changing to low-calcium Ringer the heart was at once stimulated to a high level of activity for

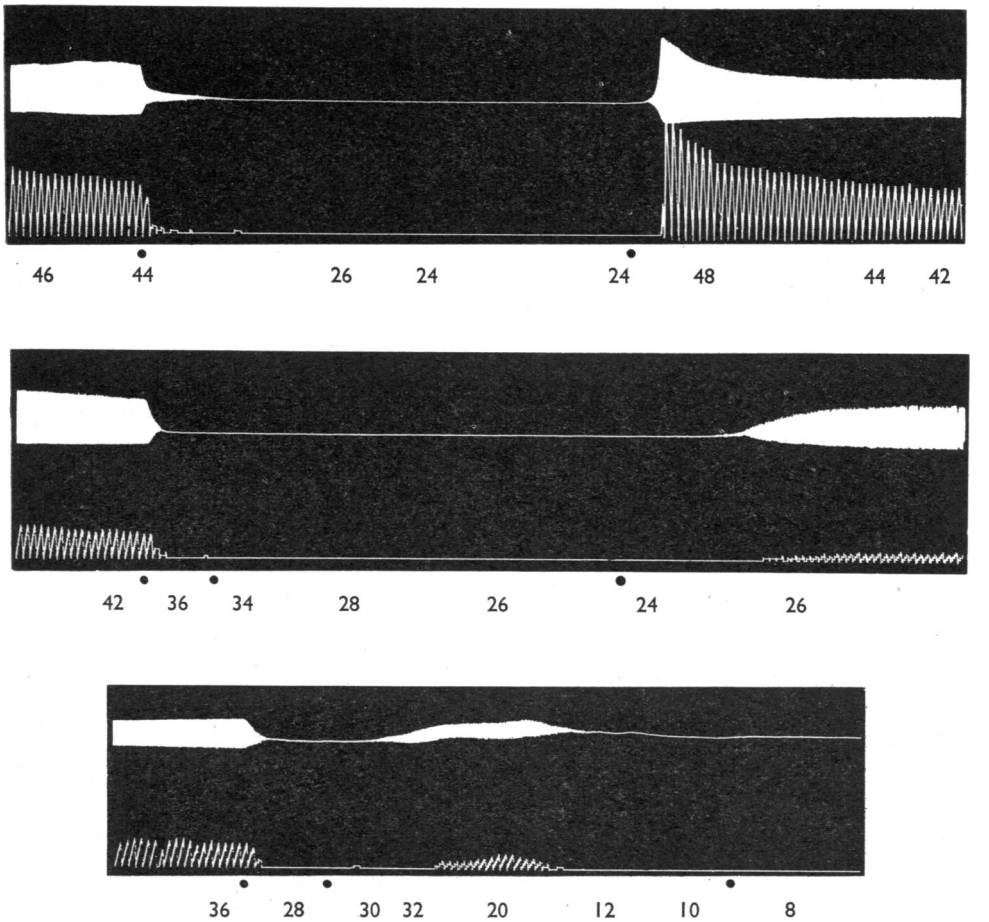

Fig. 1. *Upper tracing:* An isolated frog heart perfused with low-calcium Ringer solution. From above downwards are recorded ventricular movements (systole being shown by an upwards movement), cardiac output each 30 sec, and heart rate in beats per min. At the first dot the perfusion fluid was changed to calcium-free Ringer. At the second dot, 35 min later, perfusion with low-calcium Ringer was resumed. Twitch tension and cardiac output returned rapidly. *Middle tracing:* Heart, as before, perfused with low-calcium Ringer solution. At the first dot perfusion with calcium-free Ringer solution was started. At the second dot the calcium-free Ringer solution contained hydrogen peroxide 1×10^{-5}. The heart was not stimulated. 30 min later at the third dot perfusion with low-calcium Ringer solution was resumed. Several minutes elapsed before twitch tension returned. This and the subsequent occurrence of cardiac irregularities and bradycardia indicate that peroxide was combining with some constituent of the heart although stimulation did not occur in the absence of calcium from the perfusion fluid. *Lower tracing:* Heart, as before, perfused with low-calcium Ringer solution. At the first dot perfusion with calcium-free Ringer solution was started. At the second dot the calcium-free Ringer solution contained digitoxigenin $2{\cdot}5 \times 10^{-5}$. Digitoxigenin caused a return of twitch tension and cardiac output, though these were not increased to the levels normal for low-calcium Ringer solution. At the third dot, 30 min later, perfusion with low-calcium Ringer was recommenced, but the ventricle was arrested and the auricles alone were beating.

3 to 4 min, after which ventricular contractions, heart rate and cardiac output were similar to those at the start of the experiment (Fig. 1). Of the drugs tested ouabain and digitoxigenin regularly stimulated the hearts. The stimulation began within 8 min. The force of the ventricular beat was increased and the cardiac output recommenced, but the heart was not stimulated to the level of activity normal for low-calcium Ringer. Heart block ensued and the final change to low-calcium Ringer found the ventricle unresponsive (Fig. 1) though the auricles continued to beat. Veratridine produced a feeble stimulation in one out of three preparations. The two tannins, hydrogen peroxide, sodium oleate and sodium caprylate produced no stimulation. However, events at the change from calcium-free Ringer containing drug to low-calcium Ringer suggest that there was nevertheless some reaction with the heart. Thus, after perfusion with the tannins, sodium oleate, and hydrogen peroxide, there was sometimes a delay before the twitch tension of the heart was restored. Sometimes cardiac irregularities were seen and the cardiac output did not completely recover. On changing to low-calcium Ringer after perfusion with veratridine a period of bradycardia attended the return of twitch tension.

Antagonism of ouabain action in calcium-free Ringer. Of the drugs tested only hydrogen peroxide and the two tannins modified the stimulant action of ouabain. Prior perfusion with hydrogen peroxide for 10 min delayed the onset of ouabain stimulation for 20 min. Prior perfusion for 20 min gave almost complete protection for the whole of the 30 min and on changing to low-calcium Ringer the twitch tension and cardiac output returned. Simultaneous perfusion of hydrogen peroxide and ouabain produced the effects of ouabain alone. Perfusion with tannic acid completely prevented ouabain stimulation, but on changing to low-calcium Ringer 30 min later the heart did not beat. Tannic acid could, however, protect against ouabain stimulation for 15 to 20 min, and on changing then to low-calcium Ringer the contractile properties of the heart returned after some delay. Perfusion with paullinia tannin in calcium-free Ringer for 10 min either greatly delayed or completely protected hearts over a subsequent 30 min period of perfusion with ouabain.

Failure of ouabain to stimulate calcium-depleted hearts. Frog hearts perfused with low-calcium Ringer and then with calcium-free Ringer containing edetic acid for 30 min behaved like hearts perfused with calcium-free Ringer ; but on changing back to low-calcium Ringer there was an interval before twitch tension suddenly returned. This interval varied in different experiments from 10 to 90 min. Perfusion for 10 min with calcium-free Ringer containing edetic acid completely prevented, over a 30-min period, the action of ouabain in calcium-free Ringer containing edetic acid. On changing back to low-calcium Ringer the interval before contractility returned was as a rule shortened. The heart then showed signs of ouabain fixation.

Hearts perfused for 3 to 4 hr with calcium-free Ringer resembled hearts perfused with edetic acid in that on changing the perfusion fluid to low-calcium Ringer there was a variable interval before the twitch tension returned. If at the end of the 3 to 4 hr perfusion with calcium-free Ringer the heart was then perfused for 30 min with calcium-free Ringer containing ouabain, no stimulation of the heart occurred. When perfusion with the low-calcium Ringer solution was resumed twitch tension

returned more rapidly than in control hearts, and toxic effects attributable to ouabain, such as bradycardia and systolic arrest of the ventricle, usually occurred.

DISCUSSION

Calcium is believed to be present in the heart partly in the ionic form, but largely in some form of chemical combination (Pohle, 1935 ; Krogh, Lindberg & Schmidt-Nielson, 1944). Hajdu & Leonard (1959) suggest that calcium is involved in a normal muscle twitch response in at least three sites—at the cell membrane, which is, however, very resistant to calcium deprivation ; at a superficial site where it is required for the propagation of excitation from the cell membrane inwards ; and, finally, at the contractile protein itself which requires calcium in order to contract. Excess calcium here may cause contracture. Hearts perfused with low-calcium Ringer lose calcium from the superficial site required for the propagation of excitation from the cell membrane inwards. Loss of calcium, however, continues long after the change in twitch tension is complete (Niedergerke, 1957). In most frog hearts perfused with calcium-free Ringer the twitch tension falls to zero because of a rapid and severe depletion of this superficial calcium, but loss of calcium continues afterwards from calcium stored in the heart. In frog hearts perfused for up to 35 min in calcium-free Ringer calcium stores are not completely depleted and the twitch tension returns rapidly when perfusion with low-calcium Ringer is resumed.

Frog hearts perfused for 3 to 4 hr in calcium-free Ringer suffer a progressive loss of calcium so that stores in the heart become depleted, and calcium is also lost from some less accessible site—perhaps where it is necessary for the shortening of the contractile protein. When perfusion with low-calcium Ringer is resumed there is usually a long interval before cardiac function is restored. This interval is presumably required for the movement of calcium from the perfusion fluid back into the heart. Perfusion of frog hearts with calcium-free Ringer containing edetic acid might be expected to cause very rapid depletion of calcium ; and, in fact, the hearts behave in a fashion similar to hearts perfused for 3 to 4 hr in calcium-free Ringer, in that on changing the perfusion fluid to low-calcium Ringer there is a long latent period before cardiac function is restored. Under different experimental conditions Thomas (1960) reported that edetic acid destroyed the semipermeability of the muscle fibre membrane and this might be due to depletion of calcium in the membrane itself.

Clark (1913) and Loewi (1955) found that oleate stimulated the frog heart only when calcium was present in the perfusing fluid. This observation is confirmed and extended to cover the actions of caprylate, tannic acid, paullinia tannin and hydrogen peroxide. Hearts perfused for 5 min in calcium-free Ringer may be regarded as being deficient in the superficially located pool of calcium required for propagation of the excitatory process from the cell membrane inwards. Stores of calcium in the heart would be largely intact. It seems probable that these drugs cause the heart to increase the uptake of calcium at the heart surface and so increase the superficial calcium pool. This belief is supported by the observation that all the drugs form sparingly soluble calcium salts and all have an affinity for heart

tissue. Eventual systolic arrest of the ventricle may result from excess calcium at the pool required for shortening of the contractile protein.

The observation that ouabain and digitoxigenin regularly stimulated hearts perfused for a short time in calcium-free Ringer is in harmony with observations by Ransome (1917), Nyiri & DuBois (1930) and Friedman & Bine (1948). If, as previously indicated, the hypodynamic state of these hearts is due to loss of calcium from a superficial site in the heart, then it is likely, though there is no direct evidence on this point, that cardiac glycosides also stimulate the heart by repairing this calcium deficiency, and indeed it has long been recognized that there are resemblances between the actions of calcium and cardiac glycosides on heart muscle. Burridge (1916) suggested that cardiac glycosides increase the response of the heart to calcium. Thus perfusion in calcium-free Ringer reduces the stimulant action of ouabain, but raised calcium levels increase the toxicity of cardiac glycosides (Baker, 1947). Salter, Sciarini & Gemmel (1949) reported that each molecule of ouabain can compensate for the loss of a given number of calcium molecules in the perfusion fluid. It is therefore suggested that the prime action of cardiac glycosides is to increase the superficial pool of calcium required for the propagation of the excitatory process from the cell membrane inwards ; but, if so, at least some of the calcium may come from stores of calcium already present in the heart, since the distinguishing feature of the action of the glycosides is their ability to cause stimulation in calcium-free Ringer when the heart store of calcium is not depleted. This view has something in common with that of Pohle (1935), who believed that strophanthus caused shifts in the equilibrium between ionic calcium, calcium complexes and protein-bound calcium complexes in the heart. In addition, however, ouabain can probably facilitate the uptake of calcium from the perfusing fluid, since though it cannot stimulate hearts in which calcium stores are depleted yet the contractile response returns relatively rapidly when perfusion with low-calcium Ringer is resumed.

The prevention of ouabain stimulation in calcium-free Ringer by prior perfusion with the tannins, or with hydrogen peroxide, may mean that fixation of ouabain takes place at some protein constituent of the heart surface. It is interesting to note that, when calcium is present in the perfusing fluid, subliminal quantities of hydrogen peroxide and ouabain have a synergistic action (Giarman, 1949). This could be explained if receptors occupied by peroxide were causing increased uptake of calcium from the perfusing fluid, and the ouabain-occupied receptors were having perhaps a similar action, and, in addition, were making available calcium already present in the heart cells.

Finally, it should be pointed out that there are differences between the actions of drugs on the frog heart and the actions of the same drugs on the more complex mammalian heart. For example, hydrogen peroxide and the two tannins stimulate the frog heart but, in the main, have a negative inotropic action on the isolated mammalian heart.

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