A LIVER-FACTOR RESPONSIBLE FOR THE POTENTIATION OF THE CARDIAC ACTIONS OF DIGITOXIN*

SUMIKO FUJINO AND MAMORU TANAKA
Department of Pharmacology, Sapporo Medical College, Sapporo

Received for publication August 24, 1966

In the previous papers (1-4), it was shown that, when digitoxin is given into the animals suffering either from circulatory disturbances in liver or from liver damages, not only the lethal dose decreases but also the characteristic changes due to digitoxin in the electrocardiogram, i.e. the prolonged PQ-interval and the depressed ST-segment, appear to a lower degree, suggesting that there is an intimate relationship between the cardiac action of digitoxin and an activity of liver. In other words, these findings would imply that digitoxin undergoes a change in its molecular level or an effective substance (or substances) is added during the passage of digitoxin in liver and thus the potentiation of the cardiac action of digitoxin in question takes place.

The purpose of the present study is to confirm the previous results more precisely in a simpler system, using isolated liver of guinea pig, and to determine the nature of the above ‘liver-factor’ potentiating the cardiac action of digitoxin, which appears in connexion with the passage of digitoxin in liver.

METHODS

1. Determination of lethal doses of various solutions

Guinea pigs weighing more than 300 g, which had starved for 16-36 hours, were used. After narcotized by urethane (0.2 g/100 g of body weight), the animals were fixed in back position. Then, canules for the injection of test solutions were inserted into the jugular vein, and 30% of blood flow in liver were excluded by means of binding the right branch of the portal vein exposed under the condition of laparatomy.

After completion of the above treatments, solutions containing 1/10 of calculated lethal doses of digitoxin, of which values were obtained from the basis of both body weight and liver condition, intact or damaged, were injected through the jugular vein every 5 minutes. The injection speed was 1 ml/min. The time points of heart arrest were determined electrocardiographically, and the actual lethal doses were given as the total of the quantities of injected solution until the heart arrest. In all experiments the artificial respiration was used.

* An outline of this study was presented at the 8th Regional Meeting (North Area) (October, 1957) of and at the 32nd Annual Meeting (March, 1959) of the Japanese Pharmacological Society and was published briefly in Folia pharmaco. japon. 54, 228 (1957) and in Ibid. 55, 178$ (1959).
2. **Recording of electrocardiogram**

Electrocardiogram by bipolar lead from two points of the animals, which corresponded to the second limb lead of the human case, was recorded by means of the usual electromagnet oscillograph (Yokokawa Elect. Co. Ltd.) with vacuum-tube amplifier. Syringe needles for subcutaneous injection (gage: 1/3) were used as electrodes.

3. **Solutions (abbreviation given in this section will be used in latter sections and the illustrations, as occasion demands)**

1) **Solution a (or a):** Liver (normal)-perfusate of digitoxin solution. In a moist chamber of 39°C, 50 ml of digitoxin solution, i.e. f, of which constitution will be given in the later paragraph, were infused into isolated normal liver of the animals at a speed of 2 ml/min. The solution thus obtained were used for experiment.

2) **Solution b (or b):** Liver (poisoned)-perfusate of digitoxin solution. CCl₄ poisoning of liver was done by treating for 36 hours with a CCl₄-olive oil mixture, in which 0.7 ml of original CCl₄ fluid per 100 g of body weight were contained and the CCl₄ fluid was diluted 11 times by olive oil. The mixture was administrated into stomach of the animals through a thin polyethylene tube. Using the liver thus poisoned, perfusion of solution f, i.e. digitoxin solution, was made as in the case of normal liver.

3) **Solution c (or c):** Liver (cooled)-perfusate of digitoxin solution. Except that the temperature of the moist chamber and of the solution to be perfused was 10°C, the procedure was the same as in the case of solution a.

4) **Solution d (or d):** Kidney-perfusate of digitoxin solution. Instead of liver, kidney of the animals was used. Since the weight of kidney was about 20% of that of liver, 10 ml of solution f were perfused. For the other points, see in 1).

5) **Solution e (or e):** Mixture of liver-perfusate of Ringer solution with digitoxin. Perfusion through liver was done of digitoxin-free solution f of 50 ml, in which ethanol was contained at 10 Vol% as will be understood in the following section. After the perfusion, of which procedure was the same as in 1), digitoxin was added to the perfusate. In the solution, the concentration of the drug was 1/2 × 10⁻⁴ g/ml, so that the concentration was about the same as that in solution a.

6) **Solution f (or f):** Ringer solution with digitoxin. Stock solution of both 2 × 10⁻³ g/ml and 1 × 10⁻³ g/ml of digitoxin was prepared by adding absolute ethanol to digitoxin pulver (Merck). Solution f was a mixture of the stock solution of latter concentration with Ringer solution to contain 1 × 10⁻⁴ g/ml of digitoxin and was, therefore, of 10 Vol% ethanol. Constitution of Ringer solution used was: NaCl, 165 mM; KCl, 5.6 mM; CaCl₂, 2.6 mM; NaHCO₃, 2.4 mM; glucose, 5.6 mM.

7) **Solution g (or g):** Heat-treated solution a. Solution a was heated at 70°C for 10 minutes and then was cooled to 37°C. After the precipitate formed during the heating was removed by filtration, the proper experimentation was done.

8) **Solution h (or h):** Mixture of CHCl₃-extract of solution a with Ringer solution. One hundred ml of solution a were shaken with 600 ml of CHCl₃ for 30 minutes, accord-
According to Brown, Shepheard and Wright (5). After allowing to stand for several hours, CHCl₃ layer was evaporated to dryness under reduced pressure. To dissolve the dry residue, absolute ethanol of 9 ml was added, and then Ringer solution was given to make total volume 90 ml. The solution thus obtained was used for the proper experimentation. The reason why the total volume was not original 100 ml but reduced by 10 ml was based on exclusion both of a part of CHCl₃ layer with Ringer droplets and of bubbles formed.

9) Solution i (or i): Mixture of digitoxin with the Ringer layer left CHCl₃ extraction of solution a. The Ringer layer in the case of 8) was heat-treated at 70°C for 10 minutes and was, then, cooled to 37°C. The precipitate due to heating was removed by filtration. The filtrate was mixed with the stock solutions of digitoxin to make the drug concentration $1/2 \times 10^{-4}$ g/ml. For the reason for making this concentration, see in 5). A necessary volume of absolute ethanol was also added to make total concentration of ethanol in the mixture 10 VOl%.

4. Determination of potassium and calcium concentrations in the perfusates

Potassium concentration was measured flame-photometrically, and calcium concentration was determined by Clark and Collip’s method (6).

RESULTS

Part 1. Experiments to show existence of the factor

Figs. 1–4 demonstrate the results on the change in the lethal doses and in the electrocardiogram due to various conditions.

1. Lethal doses of various test solutions

In order to obtain the basis for the comparison of cardiac effects of the various test solutions at the biologically equivalent doses, namely, at the same %LD (per cent of lethal dose), a series of experiments on lethal doses, which is summarized in Fig. 1, was done at first. As seen in this figure, the lethal dose of solution a (i.e., normal liver-perfusate of digitoxin solution) is almost equal to that of solution e and is about twice that of solution f (i.e., digitoxin-Ringer mixture). Furthermore, from the comparison between f and the group-b, c, and d, it is clear that, if liver activity is low or absent, the digitoxin action is almost not reduced in spite of the passage of digitoxin through the tissue.

Fig. 1. Lethal doses of the various solutions. Each vertical bar indicates standard error of the mean. For explanation of the letters beside the abscissa, see in Methods.
2. Electrocardiogram

1) The prolongation of the PQ-interval and the atrioventricular block

In all the cases observed, the PQ-interval is prolonged with the rise of \%LD or of quantity administrated of the solutions, and at last a severe alteration of the electrogram, owing to which measurement of the PQ-interval cannot be made, or the atrioventricular block appears at time points, which are shown by arrows in Fig. 2. Fig. 3 is based on this figure and shows the increase ratio of the PQ-interval at these arrows, i.e., the maximum increase ratio. According to the figure, the test solution, which gives the largest maximum increase ratio, is solution a, i.e., the normal liver-perfusate of digitoxin solution, and the order of the grade in the increase is as follows: \( a > b \div d \div e \div c \).
2) The depression of ST-level

As shown in Fig. 4, a remarkable depression of ST-level is recognized in all experimental animals in the case of solution a, which was prepared by the use of normal liver; while, in the case of solution b, which was prepared by the use of poisoned liver, the depression appears in only 4 of 9 animals tested. In other cases, percentage, at which the depression appears, is similarly small or further reduced. The maximum depression, which is observed just before the block or the severely altered electrogram begins, is most vigorous also in the case of solution a, and its mean value is 0.105 mV. In other cases, the grade in the depression is lower and roughly similar to that of the case of solution f, which is merely the mixture of Ringer solution with digitoxin.

3) The above-mentioned results on both the PQ-interval and the ST-level would be important; because, they characterize the cardiac action of solution a, that is, only the solution is prominently capable of prolonging the PQ-interval and of depressing the ST-level as compared with the other solutions at the same %-LD. Since, as stated above, only solution a is obtained by the use of liver of normal activity and its prominent action can be recognized even in animals, in which liver is partly excluded from blood circulation (see Methods), a sufficient contact of digitoxin with normal liver cell is considered to be essential for the appearance of the action and, thus, for the formation of a factor, namely, the 'liver factor' stated in Introduction.
3. Potassium and calcium concentrations in the perfusates

Table 1 shows that calcium concentration of solution a is roughly similar to that of solution e and of solution f, while, potassium concentration of solution a is considerably higher than that of solution f, i.e., the solution without passage through liver. These results might suggest that the increase in potassium concentration is the cause for the appearance of the prominent action of solution a; in other words, potassium, which is added during the passage of solution f through liver, might be the 'factor'. Since, however, solution e, of which digitoxin is without passage through liver and of which Ringer is with passage, contains considerably higher concentration of potassium and shows at the same time no noticeable cardiac action, potassium is considered not to be the 'factor'. It can be again pointed out from these considerations that an action of digitoxin on liver cell is essential for the formation of the 'factor'.

<table>
<thead>
<tr>
<th>Ion concentration (mEq/l)</th>
<th>K</th>
<th>Ca</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ringer solution</td>
<td>4.80±0.22</td>
<td>3.80±0.15</td>
</tr>
<tr>
<td>Liver-perfusate of Ringer solution</td>
<td>7.40±0.40</td>
<td>4.50±0.25</td>
</tr>
<tr>
<td>Liver-perfusate of digitoxin solution</td>
<td>6.30±0.30</td>
<td>4.20±0.28</td>
</tr>
</tbody>
</table>

Part 2. Experiments to show heat-stability of the 'factor'

1. Lethal doses of the heat-treated solution a

The heat-treatment at 70°C for 10 minutes did not exert any significant influence on the lethal doses of solution a; namely, the lethal doses of solution g (i.e., the heat-treated solution a, see in Methods) are almost equal to those of solution a.

2. Electrocardiogram

1) The prolongation of the PQ-interval

Fig. 5 shows that the grade in the prolongation of PQ-interval is scarcely influenced by the heat-treatment at 70°C for 10 minutes; namely, the grade in the case of solution g is almost the same as that in the case of solution a.

2) The depression of ST-level

Fig. 6 demonstrates that the depression of ST-level due to solution g occurred in all animals tested; namely, the situation is the same as in the case of solution a. The mean of the maximum depression of ST-level,
of which definition has been given in Part 1, is 0.095 mV in the case of solution g, which is almost the same as that in the case of solution a (i.e., 0.105 mV).

3) From the results mentioned in Part 2, it is clear that the various phenomena (lethal doses, the prolongation of PQ-interval, and the depression of ST-interval), which are exhibited in the case of heat-treated solution a, are a duplicate of those in the case of non-heat-treated solution a, indicating that the ‘liver factor’, which exists in solution a, i.e., normal liver-perfusate of digitoxin solution, is heat-stable (70°C for 10 minutes).

Part 3. Experiments on the origin of the ‘liver factor’

A series of experiments were performed to determine whether the ‘factor’ is a metabolite of digitoxin or not.

1. Lethal doses

For the same purpose as in the case of Part 1 and 2, i.e., in order to obtain the basis for the comparison between the activities of solutions to be tested, lethal doses of the solutions were first investigated. Fig. 7 shows that the doses of solution h, which is mixture of CHCl₃-extract of solution a with Ringer solution, and of solution i, which is mixture of digitoxin with the Ringer layer left CHCl₃ extraction of solution a, are similar to those of solution a, in which the ‘factor’ exists.

2. Electrocardiogram

1) The prolongation of the PQ-interval

Fig. 8 demonstrates that the maximum increase ratio of PQ-interval in the case of solution i is approximately the same as that in the case of solution a, in which the ‘factor’ exists; while the ratio in the case of solution h is considerably lower than that in the case of solution a.

2) The depression of ST-level

The results so far obtained show that solution i is more active than solution h in the point of ST-depressing action, though even solution h can depress the ST-level. The
experiments to show the ultimate relation between CHCl₃ extraction and the depression of ST-level are, however, at present in progress.

3) As is already understood, the results on the electrocardiogram, especially on the prolongation of PQ-interval, demonstrate that the 'liver factor' is water-soluble. This fact is noticeable with respect to the origin of the 'factor' and suggests that the 'liver factor' is not a known metabolite of digitoxin (5, 7--9).

FIG. 7. Lethal doses of the various solutions. Cf. Fig. 1.

FIG. 8. Maximum increase ratio of PQ-interval under the various conditions. Cf. Figs. 3 and 5.

DISCUSSION

The present results demonstrate the following several points: 1) During the passage of digitoxin through liver of guinea pig, a factor appears in the perfusate. The factor is capable of potentiating the cardiac actions of digitoxin, i.e., the prolonging action on PQ-interval and the depressing action on ST-level of electrocardiogram. 2) The factor can not be formed, unless liver is of normal state and unless the tissues perfused are liver; hence, the factor is exactly the 'liver factor'. 3) The 'factor' is water-soluble and considerably heat-stable; however, it is not such cations as potassium and calcium.

Since, as mentioned in Methods, the animals tested in the present study suffer from circulatory disturbance in liver, it is clear that the present findings does confirm precisely in a simpler and more exact system the previous findings (1--4) that, when digi-
toxin is given into the animals suffering either from circulatory disturbances in liver or from liver damages, the characteristic changes due to digitoxin in the electrocardiogram, i.e., the prolongation of PQ-interval and the depression of ST-level, appears to a lower degree.

How the 'factor' is formed is at present not understood. The necessary condition for the formation is; first, liver is sufficiently physiological; second, there must be an direct interaction between digitoxin and liver cell. Though potassium of relatively high concentration exists in liver-perfusate of digitoxin solution and the 'factor' is heat-stable and water-soluble, the 'factor' is not potassium, as written in Results. The next possibility with respect to the nature of the 'factor' is that this might be a known metabolite of digitoxin (5, 7-9); but, this possibility is also not probable; because, the 'factor' is water-soluble, as shown in Results, and the reports presented so far (5, 7-9) show that the known metabolites are not water-soluble. At present, the following possibility could be, therefore, considered: Namely, the 'factor' is a new as yet unknown metabolite of digitoxin, which is water-soluble, or is alternatively a substance, which is not any metabolites of digitoxin and is formed due to an action of digitoxin in physiologically functioning liver. These possible substances must be, of course, heat-stable.

In the present paper, two have been pointed out with respect to the actions of the 'factor': potentiation of both PQ-prolonging and ST-depressing actions of digitoxin on electrocardiogram. In addition to these, the other two actions have been already demonstrated: First, Nakano and Hayashi (10) have found that the 'factor' enhances the entrance and exit of potassium and sodium due to digitoxin in guinea pig heart; second, one of the present authors (11) has shown that shortening of shortly glycerol-extracted psoas fibers of rabbit due to adenosinetriphosphate is affected by digitoxin, if the 'factor' is present.

SUMMARY

1. The contribution of liver to action of digitoxin on heart was studied in guinea pig electrocardiographically, using the liver-perfusate of digitoxin solution, which was obtained by means of isolated guinea pig liver.

2. During the passage of digitoxin solution through isolated liver of guinea pig, a factor appears in the perfusate. The factor is capable of potentiating the cardiac actions of digitoxin, i.e., the prolonging action on PQ-interval and the depressing action on ST-level of electrocardiogram, even in the animals with insufficiently functioning liver, in which the cardiac action of digitoxin occurs usually to a lower degree.

3. The factor is formed, only under the condition of physiologically functioning liver. If liver for the perfusion is cooled or CCl,-poisoned, the formation of the factor is insufficient. The factor cannot be formed, if kidney is used for perfusion instead of liver. Hence, the term the 'liver factor' is quite justified.

4. The factor is stable to heating at 70°C for 10 minutes and is water-soluble.
Acknowledgement: The authors wish to express their gratitude to Prof. T. Tanabe and Dr. S. Kikuchi for their kind advice and encouragement.

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