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PRESENCE OF DIGITALIS-LIKE FACTOR IN MAMMALIAN PLASMA

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SUMMARY: We attempted to purify a digitalis-like factor from volume expanded dog plasma using an inhibitory effect on the binding of [^{3}H]ouabain to intact human erythrocytes to monitor digitalis-like activity. A highly polar [^{3}H] ouabain displacing compound was purified to a high degree using a combination of chromatographic procedures including reverse phase and gel filtration high performance liquid chromatography. This compound, a reversible inhibitor of [^{3}H]ouabain binding, closely resembles ouabain in its polarity and significantly increases during saline infusion. Its molecular weight was estimated to be 343Da. Moreover, similar compound was consistently detected in other mammalian plasma.

Endogenous digitalis-like factor supposed to be the natural ligand for the digitalis receptor of the Na^+,K^+-ATPase(E.C.3.6.1.3.) may be implicated in the regulation of sodium excretion and the pathogenesis of human essential hypertension (1-4). Despite widespread efforts, isolation and identification of this factor have been difficult to date. The main reason is the limited specificity of assays available to monitor digitalis-like activity.

We assumed that a natural ligand for the digitalis receptor should be searched for on the basis of the effects on intact cells. Recently, we found that an inhibitory activity on [^{3}H]ouabain binding to intact human erythrocytes is the most sensitive and relatively specific method to determine digitalis-like activity and purified a highly polar digitalis-like factor to a high degree from human urine using this method (5). This compound acted on cultured rat vascular smooth muscle cells as an inhibitor of Na^+,K^+-ATPase.

We report here the purification of digitalis-like factor from volume expanded dog plasma. Furthermore, we confirmed the presence of similar compound in other mammalian plasma.

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MATERIAL AND METHODS: Purification of [^{3}H]ouabain displacing compound. Blood was obtained from thirteen pentobarbital-anesthetized dogs in which 0.9% NaCl solution (10% of body weight) was infused over a 60-min period. The separated plasma was kept at -20°C until use. 10 litres of plasma were processed in a similar way to human urine as previously described (5). The separation steps by ion exchange resins were omitted. Throughout the purification steps, an inhibitory activity on [^{3}H]ouabain binding to human erythrocytes was employed to determine digitalis-like activity according to the method previously described (5). First, dog plasma was treated with Amberlite XAD2 preequilibrated with distilled water. [^{3}H]ouabain displacing compound (ODC) was eluted with methanol and evaporated to dryness. The residue was dissolved in 0.1M acetic acid and fractionated with a Sephadex G25 column (2.6×40cm) in 0.1M acetic acid at a flow rate of 6ml/hr. The ODC was found to reside in a fraction at elution volume between 130 and 160ml. The active fractions coming from gel filtration were applied to Sep-Pak C18 cartridges. The ODC was eluted with 20% acetonitrile and lyophilized. The active material was subjected to the reverse phase HPLC on an AM302 C18 column (YMC, Kyoto, Japan; 0.46×15cm) eluted with a gradient of acetonitrile (0-20%) over 20 min at a flow rate of 1 ml/min. The ODC was rechromatographed on an AM302 column eluted with 8% acetonitrile at 1 ml/min. The active fraction was further chromatographed by gel filtration HPLC on a diol-60 column (YMC; 0.08×50cm) in 0.1M phosphate buffer, PH 7.4/acetonitrile (80/20) at 1 ml/min.
Polarity and molecular weight of ODC. Polarity of ODC was estimated from the elution position on an AM302 C18 reverse phase HPLC column in comparison with ouabain, digoxin and aldosterone. Molecular weight of ODC was estimated from the elution position on a diol-60 gel filtration HPLC column in comparison with angiotensin II (MW=1046), ouabain (584) and taurine (125). ODC was analysed by fast atom bombardment mass spectrometry (Nihon Denshi; DX-300).
Reversibility of action. Red blood cells were preincubated at 37°C for four hours in the presence of ODC which inhibits [^{3}H]ouabain binding by 50%. Then red blood cells were washed three times with ODC-free buffer and divided into two parts. One part was incubated in the absence of ODC and the other in the continuous presence of ODC for further four hours. Thereafter, specific binding of [^{3}H]ouabain to red blood cells was assessed.
Effect of volume expansion. The concentrations of ODC were determined before and after acute saline infusion in blood samples (40ml) taken from five dogs. The concentration of ODC was expressed as "ouabain equivalents" by comparing the [^{3}H]ouabain displacement with that of a known concentration of ouabain.
Presence of ODC in other mammalian plasma. To investigate the possible existence of similar [^{3}H]ouabain displacing compound in other mammalian plasma, we collected approximately 100ml each of human, bovine and rat plasma. These plasma samples were processed identically to the volume expanded dog plasma using the chromatographic systems outlined above.

RESULTS: A crude ODC was obtained by a combination of adsorption chromatography, gel filtration and Sep-Pak C18 cartridges. Figure 1A shows the chromatographic pattern of the separation of crude ODC on an AM302 C18 column using a linear gradient of 0-20% acetonitrile in distilled water. The main peak with digitalis-like activity appeared at a retention time of 18min. This active fraction was further analysed by HPLC on an AM302 column using an isocratic elution of 8% acetonitrile. The main active peak emerged at a retention time of 15min. Figure 2A depicts the elution profile of ODC on a diol-60 gel filtration HPLC column. A single active fraction with no apparent UV absorption at 214nm was eluted at a retention time of 17 min. On the assumption that recovery of ODC through the purification steps was 100%, the concentration of ODC was estimated to be approximately 200pM in volume expanded dog plasma.

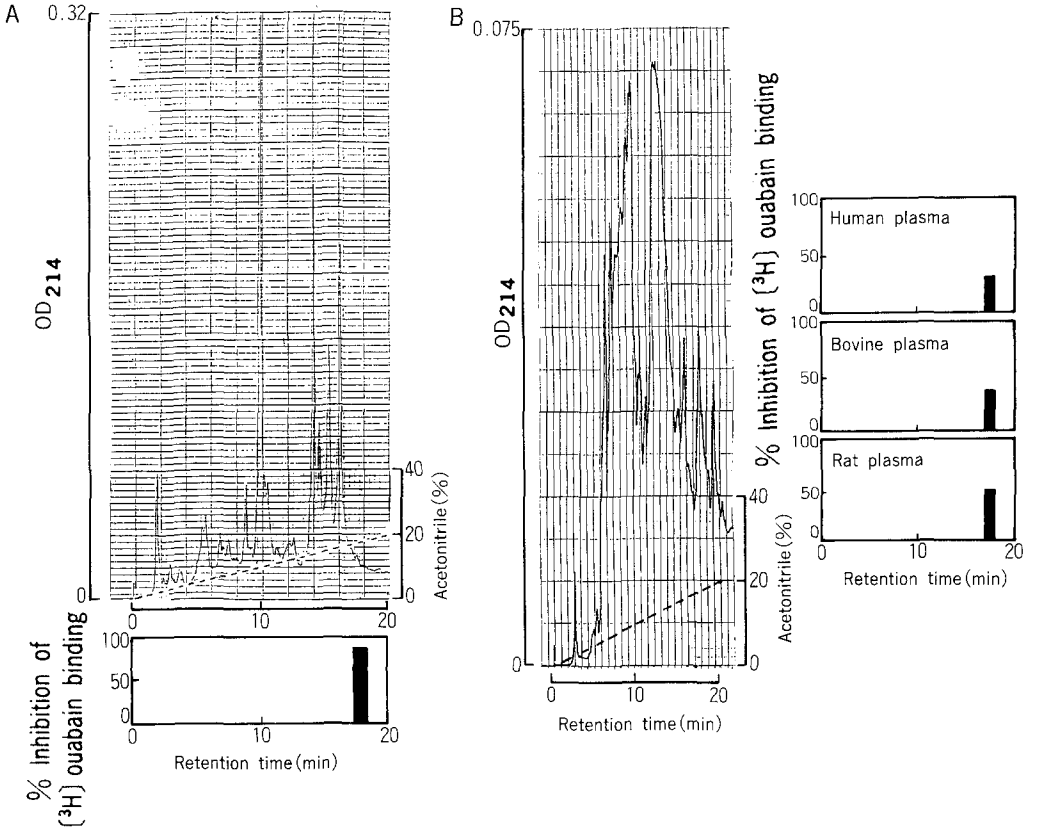

FIGURE 1: A: Elution profile of [^{3}H]ouabain displacing compound from volume expanded dog plasma on an AM 302 C18 column. The active fractions coming from previous steps were analysed by reverse phase HPLC using a linear gradient of acetonitrile (0 - 20%) over 20 min at a flow rate of 1 ml/min. The optical density was monitored at 214nm. One min fractions were collected, freeze-dried and assessed for their capacity to inhibit [^{3}H]ouabain binding to human erythrocytes. B: Elution profile of [^{3}H]ouabain displacing compound from human, bovine and rat plasma on an AM 302 C18 column under the same conditions described in A. The chromatogram shown was obtained with 100 µl sample corresponding to 50ml bovine plasma. One min fractions were collected, lyophilized, concentrated to 1/5 of their original volume and assayed.

Authentic ouabain, digoxin and aldosterone were analysed by reverse phase HPLC on an AM303 column with a gradient of acetonitrile (0-50%) over 50 min at 1 ml/min. The retention times of these substances were 19, 35 and 31min, respectively. The elution position of ODC indicates that the polarity of ODC is quite similar to that of ouabain. ODC appears to be more polar than digoxin and aldosterone. The retention times of angiotensin II, ouabain and taurine on a diol-60 gel filtration column were 15, 16 and 17 min, respectively. According to the calibration curve between molecular weight and elution volume, the molecular weight of ODC was estimated to be less than that of ouabain (Figure 2B). The molecular mass was actually determined by FAB-mass spectrometry to be 343Da.

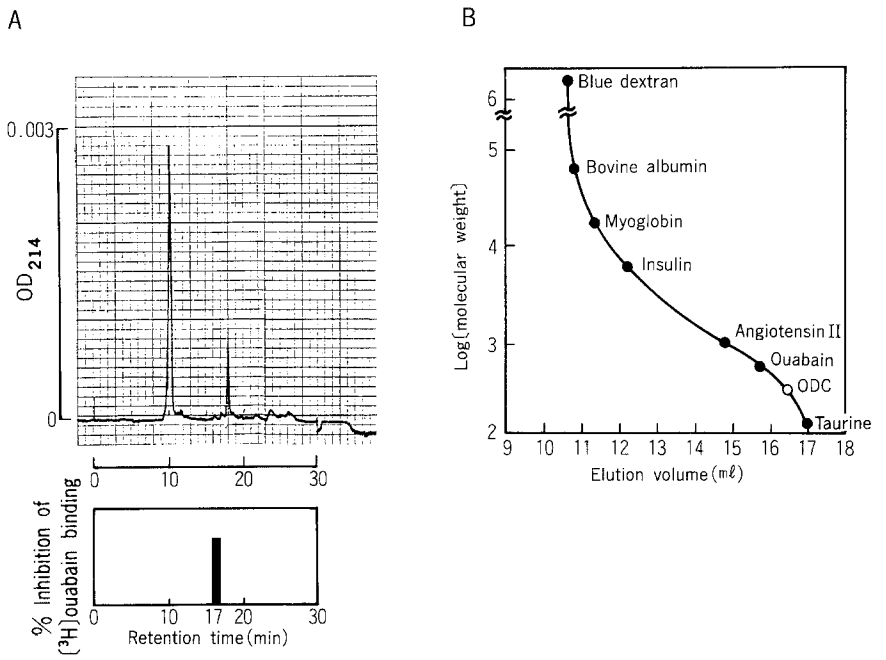

FIGURE 2: A: Elution profile of [^{3}H]ouabain displacing compound from volume expanded dog plasma on a diol-60 column. The active fraction coming from previous steps was fractionated by a gel filtration HPLC using an isocratic elution of 0.1M phosphate buffer, pH7.4/acetonitrile (80/20) at 1 ml/min. The optical density was monitored at 214nm. B: Calibration curve indicating the relationship between elution volume and molecular weight on a diol-60 column. [^{3}H]ouabain displacing compound (ODC) eluted at a position between ouabain and taurine. Each point represents the average elution position from three separate experiments with the indicated molecular weight markers.

Plasma concentration of ODC (determined at the completion of fractionation by Sephadex G25) significantly increased from 40±20 (SD)pM to 320±50 pM in five dogs during saline infusion ($p<0.01$).

Specific binding of [^{3}H]ouabain to red blood cells which had been preincubated in the presence of ODC significantly increased from 15.6±1.1 fmol/10^8 cells to 26.2±2.3 fmol/10^8 cells during four hours' incubation in the absence of ODC ($n=4$, $p<0.01$). This finding indicates that ODC may dissociate slowly from its binding site after washout and its action is reversible (Figure 3).

Figure 1B demonstrates the elution profile of [^{3}H]ouabain displacing activity in human, bovine and rat plasma by reverse phase HPLC on an AM302 column eluted with a linear gradient of acetonitrile (0-20%) over 20 min at a flow rate of 1 ml/min. ODC was consistently found in these mammalian plasma samples and showed exactly identical elution pattern to that of volume expanded dog plasma. The concentration of ODC was calculated to be approximately 40-80pM.

DISCUSSION: The possibility that endogenous inhibitors of the sodium pump exist and bind to the cardiac glycoside binding site on Na^+,K^+-ATPase has been

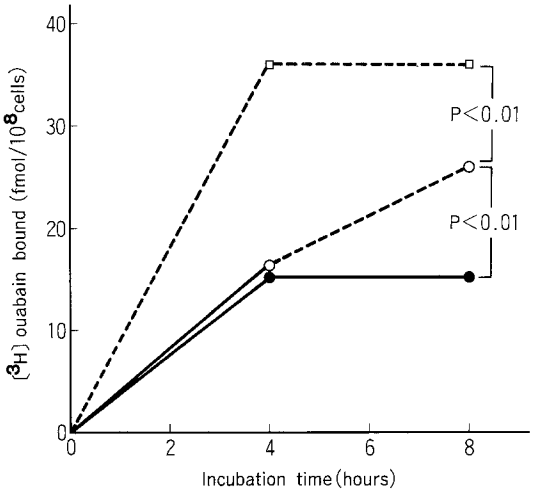

FIGURE 3: Reversibility of the action of [^{3}H]ouabain displacing compound (ODC). Red blood cells were preincubated in the presence of ODC at 37°C for four hours. Then they were washed with ODC-free buffer and incubated in the absence of ODC for further four hours. Specific binding of [^{3}H]ouabain binding to these cells (o) was compared with that to the red blood cells which were incubated in the continuous presence (●) or absence (□) of ODC. Data are mean of quadriplicates from representative experiments. The specific binding to the red blood cells (□) incubated in the continuous absence of ODC was 37.9±1.9 fmol/10^8 cells.

of much interest. However, definitive proof for the existence of endogenous ligands for the digitalis binding site remains elusive.

In the current study, we purified a highly polar ODC from volume expanded dog plasma. This compound showed exactly identical elution profile throughout the purification steps to that of urine-derived ODC we previously reported on (5). Furthermore, similar ODC was consistently found in human, bovine and rat plasma.

Several substances have been proposed as endogenous digitalis-like factors, including unsaturated fatty acids, lysophospholipids, dehydroepiandrosterone sulfate and ascorbic acid (6-9). However, none of these substances appear to be the natural ligands of the digitalis receptor of Na^+,K^+-ATPase because of their limited affinity and specificity. These compounds have no appreciable effects on our assay system as detailed elsewhere (5). The elution patterns of these substances by reverse phase HPLC are totally different from that of plasma-derived ODC. These findings strongly indicate that ODC we isolated in this study is likely to be a hitherto unrecognized, novel digitalis-like factor.

Plasma-derived ODC increased 8-fold following saline infusion. Its molecular weight was less than 500Da. The inhibitory action of this compound on [^{3}H] ouabain binding to human erythrocytes was reversible. Plasma-derived ODC resembles ouabain in its polarity and may be highly water soluble. These observations collectively suggest that the [^{3}H]ouabain displacing activity we

purified from mammalian plasma (and human urine) may be the natural ligand of the digitalis receptor and fullfil some of the criteria for the putative "natriuretic hormone" (10). Definitive conclusion must await the chemical identification of this compound which is now in progress in our laboratory.

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