Synthesis and Positive Inotropic Effect of Strophanthidol 3-Bromoacetate-19-H^3 [1]

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Strophanthidin 3-bromoacetate (SinBA) and strophanthidol 3-bromoacetate-19-H^3 (SolBa-H^3) were synthesized in order to establish their pharmacological activity as well as to determine uptake and retention of these drugs in the heart. Both drugs were found to have a rapid onset of action in guinea pig atria, reaching maximal positive inotropic effect within 20 min. SinBA was equally potent with ouabain, but ouabain was approximately 20 times more effective than SinBA. In addition, the positive inotropic effect of SinBA after a 3-hr exposure is considerably less persistent than that of the short acting cardiac glycoside ouabain. After a 3-hr exposure to SolBA-H^3 followed by a 2-hr period of contraction in drug-free buffer, the atria still contained SolBA-H^3 at a level approximately 6 times higher than the concentration of SolBA in the original buffer. Therefore, it appears that for the alkylating cardiac steroids there is a dissociation between persistence of the drug and persistence of their pharmacological effect. Finally, comparison of the known irreversible inhibitory effect of SinBA on cardiac Na-, K-ATPase with the demonstrated very short duration of its positive inotropic effect indirectly suggests that inhibition of the enzyme may not be responsible for the positive inotropic action of cardiac steroids.

Recently Hokin, *et al.*,[2] synthesized a series of alkylated cardiac steroids, the strophanthidin 3-haloacetates. They showed that these alkylated strophanthidin 3-haloacetates have the ability to irreversibly inhibit Na^+-, K^+-activated ATPase of brain. These investigators proposed that the ability of the cardiac steroids to irreversibly inhibit brain transport ATPase is due to alkylation of the enzyme by the drug.

Currently one of the favored theories for the mode of action of cardiac steroids is based on their ability to inhibit cardiac Na^+-, K^+-activated ATPase.[3] On the possibility that strophanthidin haloacetates may also irreversibly inhibit cardiac transport ATPase, which may be related to their positive inotropic action in the heart, the onset and duration of action of the alkylated cardiac steroids were investigated. Since the strophanthin series of cardiac steroids has a very short onset and duration of action, it was considered of interest to determine whether the alkylated cardiac steroids still retain their ultrashort duration of action or have an irreversible positive inotropic effect.

The following alkylated cardiac steroids, strophanthidin 3-bromoacetate (SinBA), strophanthidol 3-bromoacetate (SolBA), and strophanthidol 3-bromoacetate-19-H^3 (SolBA-H^3) were prepared, and the synthetic steps are described in the Experimental Section.

Pharmacological Procedures

Pharmacological Studies in Rabbit and Guinea Pig Isolated Atria.—Male albino rabbits and guinea pigs were used in all experiments. The animals were decapitated (rabbits) or stunned (guinea pigs), the hearts excised, and the left atria dissected. The left atria were placed in a muscle bath containing 20 ml of bicarbonate buffer[4] maintained at 30°, and oxygenated by a continuous stream of 95% O_2– 5% CO_2. The atria were stimulated electrically by a Grass Model S4 stimulator at a voltage 10% above threshold and at a frequency of 120 beats per min. Isometric contractile force was measured using a force displacement transducer and was recorded by a Grass polygraph. A resting tension of 1 g was maintained throughout the experiment. Following an equilibration period of 60 min the respective cardioactive drug was added to the bath media in either 50 μl of DMF for SinBA and SolBA or in 100 μl of H_2O for ouabain. Atria receiving the same amount of DMF as well as untreated hearts served as controls. In experiments studying the rate of washout of the pharmacological agent or its pharmacological effect, the preparations, after exposure to the drug, were washed six times by overflow and then allowed to contract in drug-free buffer for various periods of time. Unless otherwise stated, the bath fluid was replaced every 60 min thoughout the entire experiment. It was established that after 60 min of contraction complete equilibrium had been reached for the atria and, therefore, all subsequent changes in contractile force were expressed relative to the contractile force 60 min after initiation of contraction ($c + 60$).

Radioactivity Assay of SolBA-H^3 in Atrial Tissue.—In the experiments using SolBA-H^3, after exposure to the drug, the atria were washed six times and allowed to contract in drug-free buffer for 120 min. At that time the myocardial tissue was removed from the bath, blotted between absorbent tissue, weighed, and transferred into a counting vial containing 1 ml of Nuclear Chicago Solubilizer (NCS). Digestion of the tissue was achieved by leaving the closed vial for 4 hr in a water bath of 50°. This treatment yielded a transparent and homogenous solution. At this point 3 ml of absolute EtOH and 15 ml of counting solution (3 g of PPO/l. of toluene) were added and the mixture was counted using internal standardization in a Packard liquid scintillation counter. To reduce chemiluminescence, care was taken to acidify the final counting mixture by addition of 4 drops of concentrated HCl to each vial.

Results and Discussion

In order to establish the potency of SinBA a cumulative dose–response curve was obtained in rabbit atria

(1) This investigation was supported by Public Health Service Research Grant No. HE-08749 from the National Heart Institute.

(2) L. E. Hokin, M. Mokotoff, and S. M. Kupchan, *Proc. Nat. Acad. Sci. U. S.*, **55**, 797 (1966).

(3) K. Repke, *Proc. Int. Pharmacol. Meet., 1st, 1961*, **3**, 47 (1963).

(4) M. B. Chenoweth and E. S. Koelle, *J. Lab. Clin. Med.*, **31**, 600 (1946).

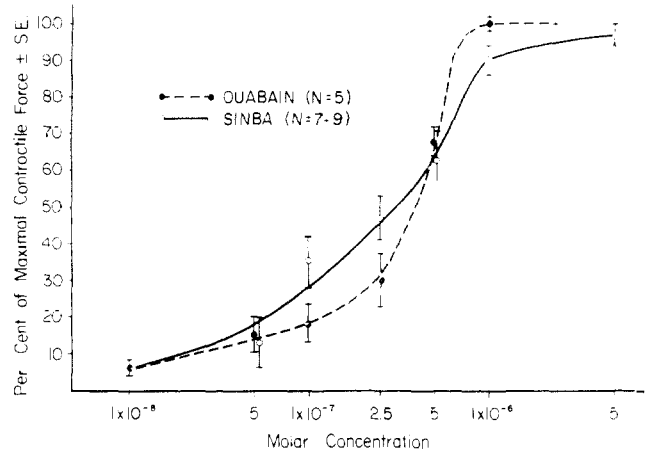

Figure 1.—Cumulative dose–response curve for SinBA and ouabain in rabbit atria.

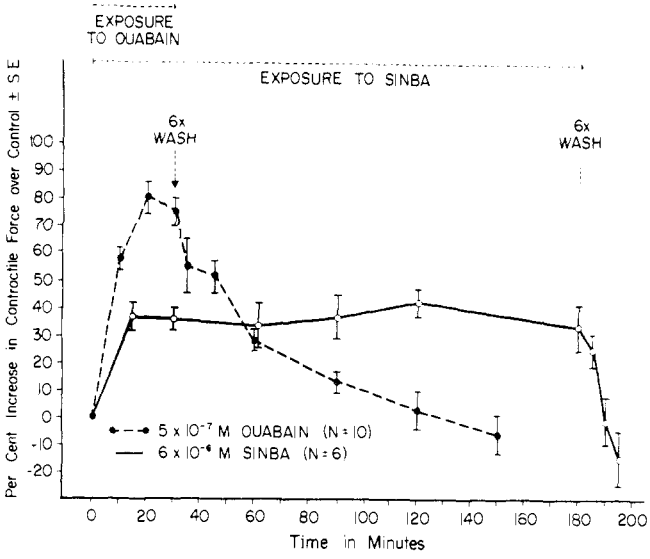

Figure 2.—Extent and persistence of the positive inotropic effect of ouabain and SinBA in guinea pig atria.

and was compared to that of the "short-acting" cardiac glycoside ouabain. In Figure 1 are shown dose-response curves for both SinBA and ouabain. As can be seen, these two cardioactive drugs are equally potent with respect to mean dose producing 50% of maximal response (ED_{50}) in rabbit atria; the ED_{50} for ouabain being 3.7×10^{-7}, the ED_{50} for SinBA being 3.3×10^{-7}. The slope, however, is less steep for SinBA than for ouabain over the dose range studied.

On the assumption that irreversible alkylation of the binding sites of cardiac receptors by SinBA may take place over a long period of time, guinea pig atria were exposed for 3 hr to maximal inotropic doses of the drug (6×10^{-6} M), then washed six times, and the persistence of the positive inotropic effect was followed by having the atria contract in drug-free buffer for an adequate length of time. The results are compared to those obtained with ouabain as shown in Figure 2. It should be noted that although the dose of ouabain is ten times less than that of SinBA, its positive inotropic effect is approximately twice as large. Both drugs demonstrate a rapid onset of action reaching maximal effect within 20 min. Thus, although the

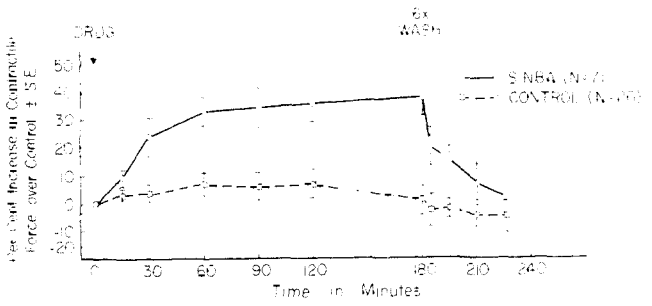

Figure 3.—Extent and persistence of the positive inotropic effect of SinBA in rabbit atria.

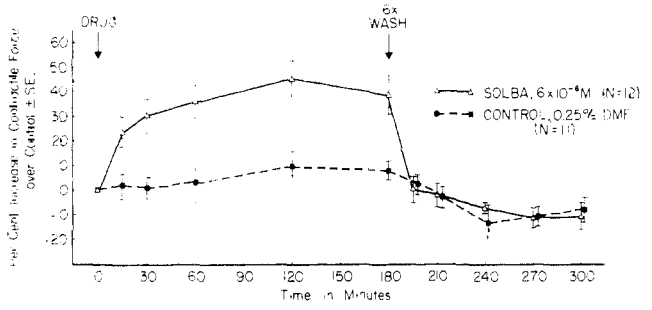

Figure 4.—Extent and persistence of the positive inotropic effect of SolBA in rabbit atria.

two drugs are equally potent (Figure 1), ouabain is considerably more effective than SinBA. When both groups of atria were washed six times and allowed to contract in drug-free buffer for various periods of time, the positive inotropic effect of ouabain disappeared between 60 and 90 min after washout ($t_{1/2}$ = 24 min), whereas the increase in contractile force due to SinBA disappeared within 10 min after commencement of washing. Thus, the positive inotropic effect of SinBA after a 3-hr exposure is considerably less persistent than that of the short acting cardiac glycoside ouabain. In order to establish that this phenomenon was not species-dependent, rabbit atria were similarly exposed for 3 hr to a dose of 5×10^{-6} M SinBA. As can be seen in Figure 3, onset, extent, and washout of the positive inotropic effect are essentially the same in rabbit as in guinea pig atria ($t_{1/2}$ = 12 min for SinBA in rabbits).

In order to compare the short persistence of the positive inotropic effect of the alkylated cardiac steroid to the retention of the drug itself, radioactively labeled SolBA-H^3 was used to follow its persistence in the heart. Figure 4 shows the effect of SolBA on the contractile force of rabbit atria. SolBA is equally effective as SinBA, and the onset and washout of the positive inotropic effect are also of the same order of magnitude. Within 30 min after washout no difference in positive inotropic effect can be seen between SolBA-treated and control atria. All atria were allowed to contract for an additional 90 min in drug-free buffer and at the end of this period the content of SolBA-H^3 was determined in the atria. The buffer concentration during the 3-hr exposure was 2.24×10^6 dpm/ml (6×10^{-6} M SolBA-H^3). After washing and contraction in drug-free buffer for 120 min the concentration in the atria amounted to 12.6×10^6 dpm/g (3.39×10^{-5} M). Thus, at the end of the 2-hr washout period the radioactivity in the atria is still approximately six times

higher than the original concentration of the buffer during exposure to SolBA-H[3]. As mentioned earlier Hokin, *et al.*,[2] have demonstrated the irreversible inhibitory action of alkylating cardiac steroids on brain Na-, K-ATPase. We have also reported similar irreversible inhibition of SinBA on rabbit heart Na-, K-ATPase in a preliminary report[5] (a detailed report will be published elsewhere). However, as indicated in the present study the positive inotropic effect of SinBA and SolBA are readily reversible and have a very short duration of action. Therefore, there appears to be a dissociation between inhibition of Na-, K-ATPase and the positive inotropic effect of SinBA. Thus, present indirect evidence suggests that inhibition of cardiac Na-, K-ATPase may not be responsible for the positive inotropic action of cardiac steroids. Furthermore, the long persistence of the drug in the myocardium after all positive inotropic effect has disappeared following washout suggests that the drug remaining in myocardial cells is bound to nonspecific sites.

Experimental Section[6]

Strophanthidin 3-bromoacetate (SinBA) was prepared as described by Kupchan, *et al.*[7] The SinBA prepared was chromatographically identical with a reference sample kindly supplied by Professor S. Morris Kupchan, mp 190–193°. *Anal.* ($C_{25}H_{33}BrO_7$): C, H, Br.

Strophanthidol 3-Bromoacetate (SolBA).—A solution of SinBA (0.500 g, 0.95 mmol) in purified dioxane (25 ml) was treated with $NaBH_4$ (0.32 mmol) and H_2O (0.5 ml). The mixture was then stirred at room temperature for 6 hr. Tlc was used to fractionate and identify the reaction products. The tlc consisted of silica gel G as the absorbent and EtOAc (system I) and $CHCl_3$–EtOH (6:1) (system II) as the solvent systems. Prior to use, the tlc plates were activated by heating for 30 min at 110°. The spots were identified by spraying the plates with a solution of 10% phosphomolybdic acid in MeOH and were heated for 5–10 min at 110° to locate the spots. Using the tlc systems the presence of four substances was noted and these were tentatively identified as strophanthidin 3-bromoacetate, strophanthidin, strophanthidol 3-bromoacetate, and strophanthidol. The reaction mixture was diluted with H_2O (25 ml) and the *dioxane was removed rapidly below 25°*. The crystalline product formed during the removal of the dioxane was collected and dried over P_2O_5 (yield 0.38 g). Tlc indicated that the material was a mixture of SinBA and SolBA with only a trace of the more polar decomposition products. Chromatography on silica gel followed by repeated crystallization from Me_2CO–petroleum ether gave 65 mg (13%) of SolBA, mp 206–208°. *Anal.* ($C_{25}H_{35}BrO_7$): C, H, Br.

Strophanthidol 3-Bromoacetate-19-H[3] (SolBA-H[3]).—A solution of SinBA (25 mg, 0.048 mmol) in purified dioxane (2 ml) and H_2O (50 μl) was mixed with the contents of a vial of tritiated $NaBH_4$ (6.7 Ci/mmol, total activity 100 mCi, equivalent to 0.015 mmol $NaBH_4$). The mixture was shaken at room temperature for 5 hr, diluted with H_2O saturated with NaCl (10 ml), and quickly extracted with $CHCl_3$ (3 × 25 ml), and then dried (Na_2SO_4). The $CHCl_3$ was removed under reduced pressure and the resulting residue was redissolved in MeOH and induced to crystallize by the addition of H_2O. The crystalline product was dried and then applied, as a MeOH solution, to two 20 × 20 cm tlc plates. The chromatograms were developed with EtOAc. Autoradiography (2 hr) indicated the presence of one major band corresponding to SolBA as well as several minor more polar bands. The major band was eluted with MeOH and after crystallization gave 2.93 mg of radioactive material (specific activity 3.18 mCi/mg). The radioactive material was then dissolved in DMF and reserved for future use. An aliquot of this solution was then added to a methanolic solution of nonradioactive SolBA (20 mg) and crystallized to constant count to yield strophanthidol 3-bromoacetate-19-H[3] (8.2 mg, specific activity 73 μCi/mg).

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(6) Where analyses are indicated only by symbols of the elements or functions, analytical results obtained for those elements or functions were within ±0.4% of the theoretical values.

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Hypocholesteremic Agents. I. Substituted Stilbazoles and Dihydrostilbazoles

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The clinical use of estrogens to lower serum cholesterol levels in the human male is limited because of the undesirable hormonal effects of this group of compounds. In attempts to chemically modify the structure of diethylstilbestrol, a series of substituted stilbazoles and dihydrostilbazoles were prepared and examined for their hypocholesteremic and estrogenic properties. Maximum separation of these biological properties was observed in the dihydrostilbazole series containing small alkyl groups on both carbons of the ethylene bridge. Other structure–activity relationships in this series are discussed.

Several groups of workers[1] have reported that estrogens lower serum or plasma cholesterol levels in humans. The clinical use of estrogens for lowering the elevated serum cholesterol levels in the human male is limited, however, because of the adverse endocrinological effects of these agents. In an attempt to synthesize compounds related to diethylstilbestrol in which the hypocholesteremic activity is dissociated from the estrogenic activity, a series of substituted stilbazoles (I) and dihydrostilbazoles (II) were prepared by the methods shown in Chart I.

2- or 3-pyridyllithium was added to a substituted ketone of type III to produce a racemic mixture of the *erythro* and *threo* forms of carbinol IV. In the majority of cases (Table I) the stereoisomers were not separated

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