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Characterization of an angiotensin II-fluorescamine derivative

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The coupling of fluorescamine (4-phenylspiro[furan-2(3H),1′-phthalan]-3,3′ dione) to angiotensin II to form a fluorescent derivative was studied. Complete reaction of the peptide below concentrations of 10^{-4}M could be achieved with a fluorescamine concentration of 0·3 mg ml^{-1} of acetone at pH 8·3, and the lowest concentration detectable by fluorescence spectroscopy was 100 pmol ml^{-1}. The derivative, as prepared did not react with ninhydrin, and no fluorescence was generated when fluorescamine was reacted with (1-Sar)-AT_{II}. These data suggest that fluorescence is generated only through the coupling of fluorescamine to the *N*-terminal primary amine of AT_{II}. The AT_{II}-fluorescamine derivative has the same intrinsic activity on the contraction of rat colon (elevenfold loss of affinity), and on the release of fluorogenic corticosteroids from bovine adrenal cortical slices (sixfold loss of affinity) compared to AT_{II}. Water-hydrolysed fluorescamine and Asp-fluorescamine did not contract rat colon preparations; the contractile response to AT_{II}-fluorescamine was blocked by (8-Leu)-AT_{II}, a specific AT_{II} antagonist. These findings suggest that the AT_{II} fluorophore shares a common receptor site with the native octapeptide. The rate loss of biological activity of the AT_{II}-fluorescamine derivative was appreciably lower than that observed for AT_{II}. The present study suggests that the AT_{II}-fluorescamine derivative can be substituted for radioactively-labelled AT_{II} for use in a variety of applications.

Subsequent to the first report of a direct binding of isotopically-labelled angiotensin II (AT_{II}) to rat and bovine adrenal gland preparations (Goodfriend & Lin, 1969), similar observations have been reported in the same or other tissues (Lin & Goodfriend, 1970; Goodfriend & Lin, 1970; Devynck, Pernollet & others, 1974). Currently, direct studies of peptide hormone receptor interactions, generally employ [^{125}I] or [^{3}H] labelled peptides, the former being preferred because of the higher specific activities which can be generated (Roth, 1973). Isotopically-labelled hormones have some loss of biological potency compared to native peptides (Roth, 1973).

Recently, a new fluorogenic reagent, fluorescamine (Fluram, Roche), which has been reported to react specifically with primary amines to form stable fluorophors, has been described (Weigele, de Bernardo & others, 1972; Udenfriend, Stein & others, 1972a,b). In the present study, AT_{II} was reacted with fluorescamine and the chemical and biological characteristics of the derivative evaluated, in order to determine whether the derivative may be useful in studying direct binding of AT_{II} to specific receptors in the adrenal cortex.

Additional use of the derivative may be in the study of interactions between fluorescent agonists and their specific receptors (shift in the fluorescence spectrum).

Studies of this nature may further elucidate the binding mechanisms of pharmacologically active agents.

MATERIALS AND METHODS

(1-Asp,5-Ile)-AT_{II}, (1-Sar,5-Ile)-AT_{II} and (1-Asp,5-Ile,8-Leu)-AT_{II} were synthesized by the solid phase method according to Merrifield (1963) as modified by Park & Regoli (1972).

The following substances were used: atropine sulphate (Sigma Chemicals, St-Louis, Mo.); fluorescamine (4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'dione) (Fisher Scientific, Montreal, Que.); methysergide bimaleate (Sandoz, Montreal, Que.); oxprenolol hydrochloride and phentolamine hydrochloride, (Ciba Co. Ltd., Dorval, Que.). Polyphloretine phosphate (PPP) was obtained as a gift from AB LEO, Halsingborg, Sweden.

All other solvents and chemicals used were of reagent grade.

Preparation of the AT_{II}-fluorescamine derivative

The AT_{II} fluorophore was prepared by diluting 2 volumes of AT_{II} in aqueous solution, with 1 volume of phosphate buffer (0·2M) and adding with rapid mixing, 1 volume of freshly prepared fluorescamine in acetone, under the conditions described in results. Fluorescence was measured with a Zeiss spectrofluorometer at an excitation wavelength of 380 nm and an emission wavelength of 480 nm.

Characterization of the fluorescent derivative

Paper electrophoresis was carried out in a Durrum-type cell (Beckman Instruments, Palo Alto, Calif.) using the following solvent systems: phosphate buffer (0·05M, pH 8·2) or pyridine–acetic acid–water (PAW) (10:0·4:90; pH 6·3). Peptides were spotted on Whatman no. 1 paper strips, and developed at 400 V for 2½ h. The strips were then dried, scanned under ultraviolet light (350 nm) and stained with ninhydrin followed by Pauly's reagent.

The myogenic effect of the AT_{II}-fluorescamine derivative on rat smooth muscle, and the stimulatory effect on the secretory process in adrenal cortex were compared to the effects of the natural octapeptide. Contraction was studied using the rat colon bioassay described by Gagnon & Sirois (1972). Secretion of fluorogenic steroids from bovine adrenal cortical slices was studied by the method of Kaplan & Bartter (1962) modified as follows: bovine adrenals, obtained from freshly killed animals were trimmed of fat and connective tissues. The glands, in cold (4°) oxygenated (95% O_2, 5% CO_2) Krebs Ringer bicarbonate buffer containing 10mM glucose (KRBG) (Kaplan & Bartter, 1962), were demedullated and transversely sliced with a Stadie-Riggs microtome to a thickness of approximately 0·3 mm. The slices (~150 mg wet weight) were preincubated for 60 min at 37° in 4 ml of KRBG and then transferred to 4 ml of fresh medium. AT_{II} or AT_{II}-fluorescamine was added, and the incubation was allowed to proceed for 90 min with oxygenation. Incubation was terminated by Millipore filtration (0·45 μm) and the filtrate was stored at 4°.

Fluorogenic steroids were extracted in methylene chloride and measured according to the technique of Mattingly (1962), as modified by Jaanus, Roseinstein & Rubin (1970), using cortisol as a standard.

In some experiments, the rate of loss of biological activity of AT_{II} and AT_{II}-fluorescamine exposed to adrenal cortical slices was measured. Following 30 or 60 min of incubation, 2·5 ml of filtered incubation media were combined with 5 ml of ice-cold ethanol and centrifuged. The supernatants were evaporated in a vacuum, resuspended in distilled water and tested using the rat colon bioassay.

RESULTS

When AT_{II} (10^{-5} M) was reacted with fluorescamine 0·15 mg ml^{-1}, peak fluorescence was observed at a pH between 8·2 and 8·3. Subsequently, the AT_{II}-fluorophore was prepared at pH 8·3.

When the concentration of AT_{II} was varied from 10^{-7} to 10^{-4} M, with a fixed concentration of fluorescamine (0·3 mg ml^{-1}), the observed fluorescence was linear. The lowest detectable amount of AT_{II}-fluorescamine was 100 pmol ml^{-1}. A concentration of fluorescamine, exceeding 0·3 mg ml^{-1}, was required to produce maximal fluorescence at 10^{-3} M AT_{II}.

AT_{II} and its fluorescent derivative have different electrophoretic mobilities in both solvent systems used, the fluorophore being more electronegative. The relative mobilities expressed as the ratio of distance migrated by the fluorophore, relative to the distance migrated by AT_{II} under the conditions described in materials and methods are: in phosphate buffer, 0·841 $\pm$ 0·006 (s.e.m., n = 5) and in PAW, 0·842 $\pm$ 0·015 (s.e.m., n = 5). The electrophoretic technique was used to confirm whether a fluorescent reading could be correlated with the extent of AT_{II} derivatization. Whereas the AT_{II} fluorescamine spots observed were ninhydrin-negative, both the fluorescent and non-fluorescent AT_{II} spots gave a strong, positive reaction with Pauly's reagent. AT_{II} (10^{-3} M) reacted completely with the fluorogenic reagent at 1·2 mg ml^{-1}, as indicated by the single fluorescent spot recovered. At lower fluorescamine concentrations, 2 spots were observed, one of which was non-fluorescent and migrated with the AT_{II} standard.

Replacement of the *N*-terminal aspartic acid on the AT_{II} molecule, with sarcosine, prevented the formation of a fluorescent derivative as indicated by a complete lack of generated fluorescence.

The stability of the AT_{II}-fluorophore was evaluated as a function of time. The fluorescent activity of a solution containing 5×10^{-5} M AT_{II}-fluorophore, kept at 22° remains unchanged over 21 h.

In the remaining studies, AT_{II}-fluorescamine was routinely prepared as follows: 2 volumes of AT_{II} (10^{-3} M) plus 1 volume of phosphate buffer (pH 8·3) and one volume of fluorescamine (1·2 mg ml^{-1} of acetone).

The effects of AT_{II} and of the fluorescent derivative on the rat colon and on the liberation of steroids from bovine adrenal cortex slices are illustrated as dose-response curves in Figs 1 and 2, respectively. In both studies, the intrinsic activity (α^E as defined by Ariens, Simonis & van Rossum 1964) of AT_{II}-fluorescamine relative to AT_{II} was equal to 1·0, and the curves were parallel. For rat colon, the ED50s for AT_{II} and its fluorescent derivative were $2{\cdot}6 \times 10^{-8}$ M and $2{\cdot}8 \times 10^{-7}$ M respectively, which represents an elevenfold decrease in the affinity. The ED50s calculated from the curves describing the adrenal cortical studies (Fig. 2), were $6{\cdot}8 \times 10^{-7}$ M (AT_{II}) and $4{\cdot}0 \times 10^{-6}$ M (AT_{II}-fluorescamine) indicating a sixfold decrease in affinity. The loss in affinity thus appears to be similar in both the colon and the adrenal cortex.

Fluorescamine (1·2 mg ml^{-1}) hydrolysed in water in a 1 to 4 ratio had no myogenic effect on the rat colon; neither could contraction be elicited by an aspartic acid-fluorescamine derivative at 5×10^{-4} M. The specific antagonist (1-Asp,5-Ile,8-Leu)-AT_{II} (Regoli, Park & Rioux, 1973) at 5×10^{-4} M, abolished the response to AT_{II}-fluorescamine on rat colon when infused 30 min before injection of the agonist. The effect of the AT_{II} antagonist was not studied on adrenal cortical slices because of the strong agonistic properties of the compound.

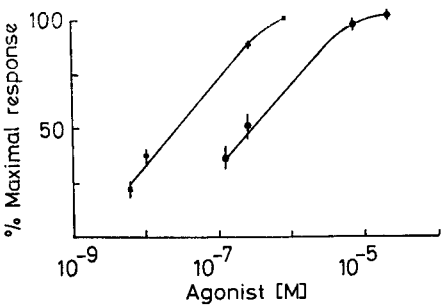

FIG. 1. Log-dose response curves of AT_{II} and AT_{II}-fluorophore as measured by the contraction of rat colon, using the maximal response elicited by AT_{II} as 100%. Procedures employed were as described in Materials and Methods. (■) AT_{II}, (●) AT_{II}-fluorescamine. Each point is the mean ± s.e.m. of 5 observations.

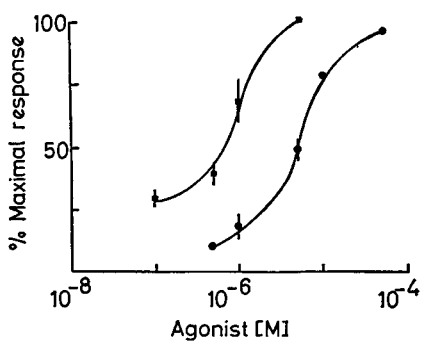

FIG. 2. Log-dose response curves of AT_{II} and AT_{II}-fluorophore as measured by the liberation of fluorogenic steroids from bovine adrenal cortex slices, using the maximal response elicited by AT_{II} as 100%. Procedures employed were as described in Materials and Methods. Each point is the mean ± s.e.m. of 4 observations for AT_{II}, and 2 observations for AT_{II}-fluorescamine except for concentrations of 10^{-5} and 5×10^{-5} M (n = 1). (■) AT_{II}; (●) AT_{II}-fluorescamine.

The rate of removal of biological activity of AT_{II} and of its fluorescent derivative, when added to suspended slices of adrenal cortex, was much slower for the derivative then for AT_{II}. More than 45% of the original activity of AT_{II}-fluorescamine remained after 1 h of incubation, compared with 8% for AT_{II} ($P < 0{\cdot}001$) [% remaining activity ± s.e.m. (n) at 30 min was for AT_{II} 28·2 ± 3·0 (4) and for AT_{II} fluorescamine 76·2 ± 4·1 (2)* and at 60 min the respective figures were: 8·0 ± 1·4 (3) and 45·8 ± 1·6 (2)* *$P = 0{\cdot}001$ compared with AT_{II}]. There was no concomitant loss of fluorescent activity.

DISCUSSION

Although the properties and characteristics of fluorophore formation can be generalized and are well documented, reaction conditions vary between individual amines and peptides (de Bernardo, Weigele & others, 1974). The pH optimum for derivative formation is such an example. In the current study, the pH optimum for the reaction of AT_{II}-fluorescamine is 8·3 which falls within the optimal pH range (8–8·5) for the combination of fluorescamine with peptides (Udenfriend & others, 1972b). The loss of linearity of fluorescence when concentrations of AT_{II} above 10^{-3} M are mixed with a fixed amount of fluorescamine (0·3 mg ml^{-1}) can be corrected by adding more fluorescamine to the reaction mixture. The maximum fluorescence observed with AT_{II} at 10^{-3} M has been shown by electrophoresis to be correlated with a 100% derivatization of AT_{II} with fluorescamine. This indirectly suggests that 100% derivatization is achieved with fluorescamine 0·3 mg ml^{-1} at concentrations of AT_{II}, below 10^{-4} M.

In the electrophoretic studies, the fluorophore was more electronegative than AT_{II} and this may be due in part to masking of the primary amine in the AT_{II} molecule by fluorescamine, as well as to the formation of a new carboxyl group in the complexed derivative (Udenfriend & others, 1972a).

Since the fluorescent derivative is ninhydrin negative, fluorescamine must be masking the *N*-terminal primary amine of the molecule. In order to verify that the coupling does not occur on the free enamine of the 2-Arg guanido group in AT_{II}, the coupling of (1-Sar)-AT_{II} to fluorescamine was tested; the secondary amine in sarcosine is unreactive toward fluorescamine (Udenfriend & others, 1972a). Unless the sarcosine residue acts directly on the conformation of the AT_{II} molecule to prevent coupling, the negative results obtained can be interpreted as proof that only the coupling of fluorescamine to the aspartic acid in position one of the AT_{II} molecule, produces fluorescence. Further confirmation was obtained from studies on the rat colon and on bovine adrenal cortical slices. Previous reports have indicated that 1-aminocyclopentane carboxylic acid (Acpc) bound to the *N*-terminal Asp of the AT_{II} molecule, reduces the affinity of the peptide by 90% in rabbit aorta (Regoli, Park & Rioux, 1974). This reduction is similar to the loss of affinity reported here for rat colon and adrenal cortex preparations in which the fluorescent derivative was tested.

The intrinsic activity of AT_{II}-fluorescamine remains unchanged compared to AT_{II} on both the myogenic and secretory processes investigated in this study and the dose-response curves for the two substances were parallel. (1-Asp,5-Ile,8-Leu)-AT_{II}, a specific antagonist of AT_{II} (Regoli & others, 1973), completely blocked the contractile response to AT_{II}-fluorescamine, suggesting that in smooth muscle, the fluorescent derivative of AT_{II} acts at the same receptor site as the native octapeptide.

After 1 h incubation of AT_{II}-fluorescamine with adrenal cortical slices, the rate of removal of biological activity of the derivative is appreciably slower compared to AT_{II} itself. Loss of biological activity of both substances may be due to biotransformation by adrenal cortical peptidases and/or to binding and uptake of the peptides (functional inactivation as has been suggested for AT_{II} by Glossmann, Baukal & Catt, 1974). Fluorescent activity does not parallel the decrease in biological activity of the derivative. The compound in this respect does not differ with other isotopically labelled hormones in which specific tests must be used to distinguish between binding to receptors and binding to metabolic enzymes.

CONCLUSIONS

The AT_{II} fluorescamine derivative has several inherent advantages over the radioactively-labelled hormone: 1) the derivative can be prepared rapidly at low cost and is stable for at least 21 h at room temperature; 2) the derivative maintains all the intrinsic activity of the native octapeptide on rat colon and bovine adrenal slices; 3) the derivative is not subject to radiolysis as are radioactively labelled hormones, and exhibits a reduced rate of loss of biological activity compared to AT_{II}.

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