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SYNTHESIS AND EVALUATION OF BIOLOGICAL ACTIVITY OF TWO NOVEL TRITIUM-LABELED DERIVATIVES OF RIODIPINE

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SUMMARY

2,6-Dimethyl-3-methoxycarbonyl-5-([2,3-$^{3}H_2$]-n-propoxycarbonyl)-4-(2'-difluoromethoxyphenyl)-1,4-dihydropyridine and 2,6-dimethyl-3,5-di([2,3-$^{3}H_2$]-n-propoxycarbonyl)-4-(2'-difluoromethoxyphenyl)-1,4-dihydropyridine were prepared by catalytic tritiation of the respective 3-allyloxycarbonyl and 3,5-di(allyloxycarbonyl) derivatives of riodipine. The two novel radioactively labeled 1,4-dihydropyridine derivatives obtained were shown to be suitable molecular tools for the identification of membrane-bound and solubilized dihydropyridine receptors associated with calcium channels in rabbit skeletal muscle.

Keywords: 1,4-dihydropyridine calcium entry blockers, ^{3}H-labeled riodipine analogues, 2,6-dimethyl-3-methoxycarbonyl-5-([2,3-$^{3}H_2$]-n-propoxycarbonyl)-4-(2'-difluoromethoxyphenyl)-1,4-dihydropyridine, 2,6-dimethyl-3,5-di([2,3-$^{3}H_2$]-n-propoxycarbonyl)-4-(2'-difluoromethoxyphenyl)-1,4-dihydropyridine, dihydropyridine receptor.

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INTRODUCTION

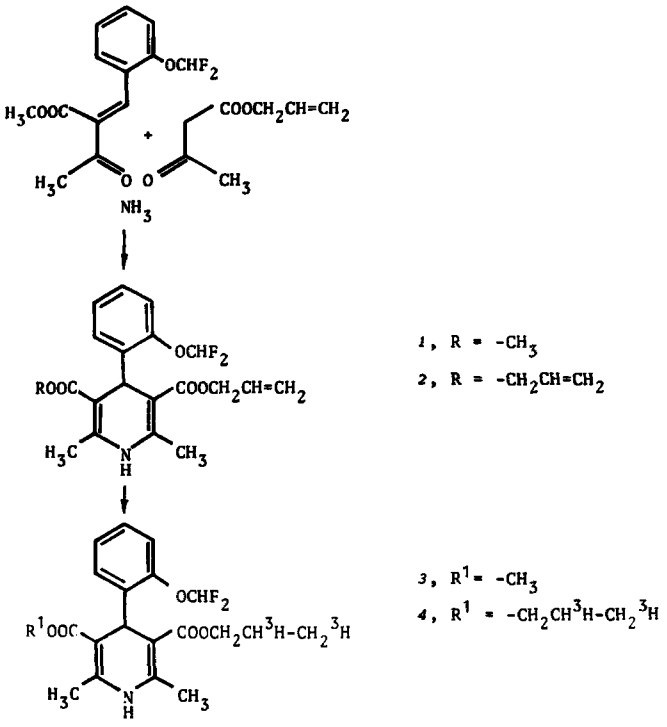
Over the last 5 years, a major investigative efforts have been focused on the application of calcium antagonists for identification and isolation of the respective receptors associated with type L calcium channels located in membranes of various nerve and muscle cells (1-3). Among these agents, dihydropyridine (DHP) calcium entry blockers represent a unique class of Ca channel probes capable of interacting with a specific binding site at nanomolar concentrations (4,5). Quantitation of these low concentrations is conveniently performed using radioisotopes. Several tritium-labeled DHPs are now commercially available including nifedipine, nitrendipine, nimodipine and PN200-110. The most widely used method for the isotopic labeling of DHPs is esterification of mono- or dicarboxylic acids obtained by the saponification of alkoxycarbonyl groups at the positions 3 and/or 5 of the dihydropyridine ring (6). However, these methods require highly radioactive substituents to be used. This step could be eliminated in the labeling technique involving exposure of unsaturated DHP derivatives to catalytic tritiation with carrier-free tritium gas.

In this paper, we present a two-step synthesis of novel radioactive derivatives of riodipine (2,6-dimethyl-3,5-dimethoxycarbonyl-4-(2'-difluoromethoxyphenyl)-1,4-dihydropyridine (7)), based on the catalytic tritiation of mono- or di(allyloxycarbonyl) congeners of riodipine provided by the Hantzsch-type cyclization. The method has the advantage of involving only one radioactive synthetic step in the preparation of DHPs with two or four tritium atoms per molecule. These derivatives were shown to be able to recognize the DHP binding sites in membrane-bound and detergent-solubilized receptor preparations.

RESULTS AND DISCUSSION

Our strategy was to introduce alkenoxycarbonyl groups at the position(s) 3 or both 3 and 5 of the dihydropyridine ring followed

by the catalytic tritiation of the DHP congeners obtained. This approach was applied to derivatives of riodipine (1 and 2) as summarized in Scheme. It involved the condensation of allyl acetoacetate



1, R = $-CH_3$

2, R = $-CH_2CH=CH_2$

3, $R^1 = -CH_3$

4, $R^1 = -CH_2CH^3H-CH_2{}^3H$

Scheme. Synthesis of tritium-labeled derivatives of riodipine.

(8), methyl 2-difluoromethoxybenzylidene acetoacetate (the synthesis will be described elsewhere) and ammonia, being primarily directed to the synthesis of the monoallyloxycarbonyl compound 1. However, 3,5-disubstituted derivative 2 and riodipine were isolated from the reaction mixture as well. This is supposed to be due to re-esterification of either the target compound 1, or starting compounds, or both. Anyhow, compounds 1, 2 and riodipine were isolated from the reaction mixture in the ratio 2.55:1.05:1.00 (by the weight), respectively. The structural assignment of these products

was established from their spectral properties, as presented in Experimental section. The formation of the compound 2 simultaneously with primary target 1 led us to pursue this seemingly undesirable reaction since it extended the number of DHP congeners up to two derivatives suitable for tritiation.

Both compounds 1 and 2 obviously are convenient precursors of radiolabeled dihydropyridine calcium entry blockers since the length of alkoxycarbonyl side chains in DHP molecule is not critical for biological activity of the drug (cf. nisoldipine (9)). Conversion of 1 and 2 to the corresponding tritium-labeled derivatives 3 and 4 was carried out using either heterogeneous (5% $Pd/BaSO_4$) or homogeneous ($(Ph_3P)_3RhCl$) catalysts. In both cases, tritiation was performed in dioxane with carrier-free tritium gas at a total pressure of 400 hPa. When the tritiation catalyzed by $Pd/BaSO_4$ was applied to 1, incorporation of two tritium atoms per molecule occured, and compound 3 was obtained in 32% yield. Again, the tritiation of 2 in the presence of $(Ph_3P)_3RhCl$ for 22 hr provided 4 in 53% yield with incorporation of four tritium atoms per molecule.

The results are indicative of the selective tritiation of C=C double bonds in 1 and 2, since the theoretical incorporation of tritium is achieved in either case. Evidently, this eliminates the possibility of the involvement of difluoromethoxy group in the reaction. Indeed, the fluorescent spectrum of 3 and 4 (wavelengthes of excitation and fluorescence emission maximum 370 and 430 nm, respectively), characteristic for riodipine derivatives (7), remained unchanged after the tritiation, thereby providing a convincing evidence for the retention of the chromophore structure of the compounds obtained.

Radioactive derivatives 3 and 4 were examined for their ability to bind specifically to DHP receptor in rabbit skeletal muscle membranes. Binding assays were performed routinely under daylight since both 3 and 4 remained stable under these experimental condi-

tions as has been shown by TLC. Both radioligands under the study bound reversibly and saturably to transverse tubule membranes (10) used to evaluate the binding characteristics.

Specific binding of the compound 4 at 1 nM to membranes was linearly proportional to concentration of protein in the assay mixture up to 30 μg/ml; thus, all binding experiments were carried out in the linear range. Kinetic binding studies made for 4 in the presence of 10 μM D-cis-diltiazem and 1 mM $CaCl_2$ (to maintain the receptor in high-affinity conformation (11)) revealed that association of the radioligand with the receptor is a pseudo-first order reaction, the k_1 value measured at 21±1°C being 0.016 $nM^{-1}min^{-1}$ (Fig. 1). The dissociation of the ligand-receptor complex followed first

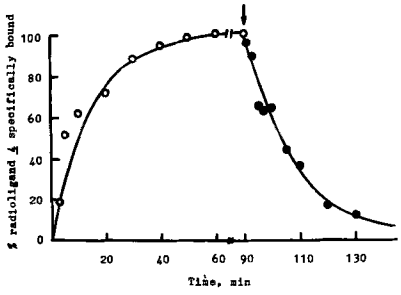


Fig.1. Association (o), and dissociation (•), kinetics of the radioligand 4 specific binding to transverse tubule membranes. Pulse-chase experiments were carried out with 1.0 nM 4 in the presence of 10 μM diltiazem and 1 mM $CaCl_2$ in the binding assay. Arrow indicates the time when 1 μM nitrendipine was added. The following rate constants were obtained after linear transformation (12) of the data from the association and dissociation reactions, respectively: k_{obs} = 0.0697 min^{-1}, k_{-1} = 0.0537 min^{-1} (correlation coefficient $r > 0.95$).

order kinetics, and the k_{-1} was 0.0537 min^{-1}. The dissociation constant (k_{-1}/k_1) calculated from these data was 3.3 nM which is consistent with the estimates of K_d from equilibrium binding experiments (Fig. 2). Scatchard analysis yielded a single class of non-

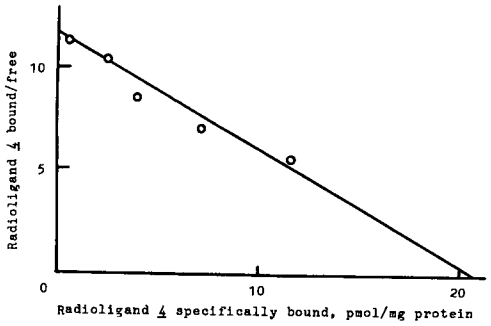


Fig. 2. Scatchard plot of the specific equilibrium binding of 4 to transverse tubule membranes. Binding assay was carried out at a protein concentration of 25 μg/ml.

interacting binding sites for the radioligand 4. Mean K_d value was 1.7 ± 0.9 nM. Nonspecific binding, measured in the presence of 1 μM nitrendipine, accounted for about 60% of the total binding, thus indicating a relatively high ability of 4 to interact with lipid core of membrane vesicles. Nevertheless, this radioligand enables the quantitation of the DHP receptor at levels as small as 3-5 fmol/assay.

Equilibrium binding studies made for 3 have demonstrated (13) that in the presence of D-cis-diltiazem and 1 mM $CaCl_2$ this radioligand, referred to further as [^{3}H]PMD, interacted with a single class of independent binding sites, the K_d value being 2.7 nM. Application of this probe allowed to detect up to 10 fmol of the DHP receptor per assay. Nonspecific binding of [^{3}H]PMD accounted for less than 15% of the total binding. These characteristics remained virtually unchanged for membranes solubilized by digitonin, so that [^{3}H]PMD was successfully applied for the identification and isolation of the DHP receptor from rabbit skeletal muscle (13).

In conclusion, the results described above clearly demonstrate the general utility of the catalytic tritiation of alkenoxycarbonyl

congeners of DHPs for preparation of radioactive probes suitable for quantitation of DHP receptors. Application of this procedure to riodipine analogs (1 and 2) produced two novel radioligands (3 and 4) which have been shown to be a potent pharmacological tools of the DHP receptor investigation.

EXPERIMENTAL

^{1}H NMR spectra were measured in deuteriochloroform on a Bruker WM-250 NMR spectrometer at 260 MHz. Chemical shifts are reported in δ units, parts per million, downfield from $(CH_3)_4Si$. Mass spectrometry was performed on Mat 44c analyzer. Ultraviolet spectra were obtained on Specord 40 spectrophotometer. Analytical thin-layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel (Merck, Kieselgel 60 F_{254}). Plates were visualized under UV light at 254 and 366 nm. Distribution of radioactivity on TLC plates was analyzed with Berthold LB 2832 automatic linear analyzer.

For radioactivity counting in dpm, samples were dissolved in Beckman Ready-Solv NA scintillation mixture and analyzed on Beckman LS 9800 liquid scintillation spectrometer at an appropriate processing mode. For radioligand binding studies, samples were routinely treated with Supersolve X scintillation cocktail (Koch-Light) and counted on Intertechnique spectrometer.

Synthesis of 2,6-dimethyl-3-methoxycarbonyl-5-allyloxycarbonyl-4-(2'-difluoromethoxyphenyl)-1,4-dihydropyridine (1) and 2,6-dimethyl-3,5-diallyloxycarbonyl-4-(2'-difluoromethoxyphenyl)-1,4-dihydropyridine (2)

A mixture of allyl acetoacetate (8) (1.42 g, 0.01 mol), methyl 2-difluoromethoxybenzylidene acetoacetate (2.70 g, 0.01 mol) and 0.01 mol ammonium hydroxide (as a 25% aqueous solution, 1.4 ml) was refluxed for 12 hr with 10 ml isopropanol. The solvent was removed on a rotary evaporator and the yellow oily residue was washed with

hexane. The titled products were isolated by the two-step preparative-scale TLC procedure. Initially, the mixture of 1, 2 and riodipine was separated from the crude product by the preparative TLC on aluminium oxide without binder (L 40/250, Chemapol) using acetone/hexane (1:1) as a developing solvent. This mixture of compounds extracted by chloroform from the spot at R_f ca. 0.5 was subsequently separated by TLC on Alufolien Kieselgel 60 (Merck, 0.20-mm layer) using benzene/diethyl ether (1:1) as a solvent. Under these conditions, riodipine, 1 and 2 were separated into three distinct fluorescent spots (R_f values 0.44, 0.50 and 0.56, respectively) visible under ultraviolet illumination at 366 nm.

Compound 1 was isolated with an overall yield 23%. Mass-spectrum, m/e 393 (M^+). ^{1}H NMR δ 2.28 (s) and 2.30 (s) (6 H, $-CH_3$), 3.60 (s, 3 H, $-OCH_3$), 4.51 (d, 2 H, $-OCH_2-$), 5.12 (d, 2 H, $=CH_2$), 5.28 (s, 1 H, C(4)-H), 5.67 (s, 1 H, N-H), 5.85 (m, 1 H, -CH=), 6.46 (t, 1 H, $-CHF_2$), 6.97 (d, 1 H, Ph-H), 7.06 (t, 1 H, Ph-H), 7.15 (t, 1 H, Ph-H) and 7.35 (d, 1 H, Ph-H).

Compound 2 was obtained with the yield 9%. Mass-spectrum, m/e 419 (M^+). ^{1}H NMR δ 2.30 (s, 6 H, $-CH_3$), 4.52 (d, 4 H, $-OCH_2-$), 5.17 (d, 4 H, $=CH_2$), 5.29 (s, 1 H, C(4)-H), 5.64 (s, 1 H, N-H), 5.82 (m, 2 H, -CH=), 6.45 (t, 1 H, $-CHF_2$), 6.98 (d, 1 H, Ph-H), 7.05 (t, 1 H, Ph-H), 7.13 (t, 1 H, Ph-H) and 7.36 (d, 1 H, Ph-H).

Synthesis of 2,6-dimethyl-3-methoxycarbonyl-5-([2,3-$^3H_2$]-n-propoxycarbonyl)-4-(2'-difluoromethoxyphenyl)-1,4-dihydropyridine (3)

10 mg of compound 1 dissolved in 0.5 ml dioxane was mixed with 5% Pd/$BaSO_4$((Serva, 10 mg) in 15-cc. Pyrex cell, then immersed in liquid nitrogen and the cell degassed up to a pressure of 10^{-3} hPa. Tritium gas was introduced into the cell at a total pressure of 400 hPa and the content was brought to room temperature. The mixture was stirred for 1 hr and thereafter the cell was frozen in liquid nitrogen, repeatedly degassed to remove the excess of tritium and

flashed with $^1H_2$. After the content was thawed, catalyst was separated by filtration and the solvent was evaporated in vacuo. Exchangeable tritium was removed by three successive evaporations in vacuo of the product which had been dissolved in 2 ml methanol. The target compound 3 was purified by TLC on Alufolien Kieselgel 60 TLC-plate developed with a mixture of benzene and diethyl ether (1:1), R_f 0.53; 32% yield. The molar radioactivity of 3 was estimated to be 2.1-2.3 TBq/mmol at 95-97% radiochemical purity.

Synthesis of 2,6-dimethyl-3,5- di([2,3-$^3H_2$]-n-propoxycarbonyl)-4-(2'-difluoromethoxyphenyl)-1,4-dihydropyridine (4).

Compound 4 was prepared in a similar fashion from diallyl derivative 2 using $(Ph_3P)_3RhCl$ (Merck) as a catalyst. In short, a mixture of 2 mg 2, 3.5 mg $(Ph_3P)_3RhCl$ and 0.3 ml dioxane was exposed to tritium gas in 15-cc. cell for 22 hr at a total pressure of 400 hPa. The titled product was isolated with 53% yield by TLC on silica gel plate, R_f 0.58 (benzene/Et_2O, 1:1). The compound 4 had a molar radioactivity of 4.1-4.4 TBq/mmol at 95-97% radiochemical purity. All the radioactive DHPs obtained were stored at -40°C as a solutions in methanol.

Binding assays

Radioligand binding experiments were performed essentially as described (13) using transverse tubule membranes from rabbit skeletal muscle (10). Assay mixtures contained 20 mM Tris-HCl (pH 7.4), 10 μM D-cis-diltiazem (Goedecke) and 1 mM $CaCl_2$. Nonspecific binding was measured in the presence of 1 μM nitrendipine (Institute of Organic Synthesis, Riga, USSR). Measurements of bound and free radioligands were done by conventional liquid scintillation spectrometry. Binding of 3 and 4 to GF/C glass fiber filters (Whatman) and assay tubes was negligible.

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