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A 62 kDa PROTEIN IS PHOTOAFFINITY LABELLED BY [^{3}H]FELODIPINE IN VASCULAR SMOOTH MUSCLE, BUT NOT IN CARDIAC AND SKELETAL MUSCLE

ANNA NILSSON, CHRISTER WESTERLUND and STIG BOSTRÖM*
Hässle Cardiovascular Research Laboratories, S-431 83 Mölndal, Sweden

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Abstract—Irradiation of a cytosolic fraction from vascular smooth muscle in the presence of [^{3}H]felodipine resulted in the labelling of a protein with an apparent molecular weight of 62 kDa. The labelling was seen on UV-irradiation at 360 nm, but not at 254, 278 or at wavelengths above 410 nm. The photolabelling was enhanced in the absence of oxygen. In cytosolic fractions prepared from porcine liver, cardiac and skeletal muscle no photoaffinity labelling of proteins between 90 and 45 kDa could be demonstrated. The results suggest that felodipine is a photoaffinity ligand and that felodipine binds to a soluble protein present in vascular smooth muscle but not in the other tissues tested.

Felodipine is an antihypertensive 1,4-dihydropyridine which selectively inhibits vascular smooth muscle in concentrations which lack significant effect on cardiac muscle [1]. The inhibition of vascular smooth muscle contraction by this type of drug is thought to be the result of membrane-related inhibition of calcium entry through voltage-operated channels.

In the rat portal vein, the electrical discharge, which is due to an inward calcium current, is inhibited by felodipine in the nanomolar range, which constitutes the clinically relevant concentration of free drug in plasma [2]. Quantitatively, the mechanical activity for a given electrical discharge is more markedly reduced owing to an apparent partial electromechanical uncoupling [3]. Thus felodipine inhibits action potential discharge, as well as a later step in the control of contraction in the rat portal vein, suggesting that the vasodilative action of felodipine may partly be due to an intracellular mechanism [1, 4].

Furthermore, felodipine, in the micromolar range, interacts *in vitro* with calmodulin [3–5] and inhibits calmodulin-dependent enzymes and processes [6]. Recent photoaffinity labelling studies have also shown binding of [^{3}H]felodipine to a 62 kDa soluble protein from porcine mesenteric vascular smooth muscle [7]. A [^{3}H]azido analogue of felodipine was found to label proteins with molecular weights of 44, 29, and 14, as well as 62 kDa [7].

In this paper we report further details of the photoactivation of felodipine and the results of the labelling experiments with soluble proteins from different tissues.

MATERIALS AND METHODS

[^{3}H]Felodipine (839 mCi/mmol) and [^{14}C]H 152/37 (56.7 mCi/mmol) were obtained from the Department of Organic Chemistry, AB Hässle, Mölndal, Sweden. H 152/37 is the oxidized pyridine analogue of the dihydropyridine felodipine.

[^{14}C]-Methylated protein standard mixture was purchased from Amersham (Amersham, U.K.) and included the following proteins with molecular weights in kDa: myosin 200, phosphorylase-b 92.5, bovine serum albumin 69, ovalbumin 46, carbonic anhydrase 30, and lysozyme 14.3. Electrophoresis reagents were purchased from Pharmacia-LKB (Uppsala, Sweden). General laboratory reagents used were of analytical grade or better and were from AB Kebo (Sweden).

Preparation of soluble proteins from vascular smooth muscle. Male and female domestic pigs, 20–25 kg body wt, were killed by an overdose of pentobarbital (Mebumal®) and exsanguinated. The small intestine was removed en bloc and prepared under refrigeration. The archade was trimmed of fat and connective tissue, dissected, rinsed and minced in 20 mM HEPES buffer, pH 7.4, supplemented with 100 mM NaCl, 2 mM EGTA and a protease inhibitor mixture (containing 40 μg/ml pepstatin, 40 μg/ml leupeptin, 6 μg/ml Trasylol®, 0.2 mM PMSF and 0.8 mM EDTA) at 4°. The minced tissue was homogenized three times for 15 sec each with a Polytron homogenizer at setting 5. The homogenate was then centrifuged at 1000 *g*, followed by 10,000 *g* for 20 min each and the supernatant was finally centrifuged at 100,000 *g* for 1 hr. The resulting supernatant was used in the photoaffinity labelling experiments. The protein concentration was measured with a dye binding assay using bovine serum albumin as standard [8].

Preparation of soluble proteins from different tissues. Porcine liver, left ventricle, and gastrocnemius muscle were dissected and minced in the HEPES–EGTA buffer as above. The fragmented tissues were homogenized as described except for liver and ventricular muscle which were homogenized once for 5 sec. The homogenates (containing about 20% tissue) were centrifuged at 14,000 *g* for 20 min and the resulting supernatants were subjected to 100,000 *g* centrifugation as above.

* To whom corespondence should be addressed.

The protein concentrations in the subsequent labelling experiments were adjusted to 300 μg/ml.

Photoaffinity labelling of soluble proteins with [^{3}H]felodipine and [^{14}C]H 152/37. Soluble proteins from the different tissues, were equilibrated for 1 hr at 37° with 6 and 12 μM [^{3}H]felodipine for total binding. Vascular smooth muscle proteins were also equilibrated with 50 μM [^{14}C]H 152/37 for total binding. Ca^{2+} was added to ensure a free Ca^{2+} concentration of 0.1 mM and 1 ml of protein solution was used. The specificity of the labelling was tested by including an excess of unlabelled felodipine. All procedures prior to photolysis were performed in dim light.

After equilibration, the samples were transferred to tissue culture multi-well plates kept on ice, and irradiated for 10 min at 366 nm (radiation peak) with a B100A BLACK-RAY® UV lamp (UVP Inc.) at a distance of 10 cm.

The irradiated samples were washed with 1 ml of the HEPES buffer with 0.1 mM Ca^{2+} and concentrated in Centricon centrifugal microconcentrators (Amicon®) with a molecular weight cut-off at 10 kDa. The proteins were separated by SDS–PAGE using a modified Laemmli [9] system with 0.7 mm thick gradient gels (7–20% acrylamide). The gels were stained with Coomassie Blue and sliced in 3 mm sections which were dissolved overnight at 80° in 0.5 ml of 33% H_2O_2.

Radioactivity was measured in a liquid scintillation counter. All slices from vascular smooth muscle were measured for radioactivity, but only from slice 11 to 20 when all the different tissues were compared. Molecular weights were determined by comparing the relative mobilities with [^{14}C]-labelled marker proteins.

Irradiation of vascular smooth muscle soluble proteins and [^{3}H]felodipine at different wavelengths. The soluble proteins prepared with EGTA from vascular smooth muscle with 12 μM [^{3}H]felodipine and Ca^{2+} added were equilibrated as described above and kept on ice until irradiation. Samples of the protein solution were then exposed to light of different wavelengths for 10 min in 2 mm quartz cuvettes containing 500 μl.

Irradiation at 254 and 278 nm was performed with a monochromator. A Corning 7-51 filter was used to yield wavelengths between 300 and 410 nm with maximum transmission at 360 nm. Photolysis at wavelengths >410 nm was achieved by filtering the light through 5 cm of saturated KNO_2 solution. The light source was a 1000 W Hg–Xe lamp.

Irradiation of air saturated and oxygen depleted sample solutions were made at each wavelength. The latter samples were obtained by bubbling the solution with argon for 1 hr.

The gels were sliced and analysed as described except that radioactivity was measured in slices 11 to 20.

RESULTS

UV-irradiation at 360 nm of [^{3}H]felodipine and soluble proteins prepared from vascular smooth muscle resulted in the incorporation of [^{3}H]felodipine into a 62 kDa protein (Fig. 1). This incorporation was seen in 23 experiments, 1291 ± 930 dpm above background (mean ± SD) with a range from 346 to 3567 dpm. Background levels were normally between 50 and 150 dpm. The molecular weight 62 ± 6 kDa is a calculated mean of 11 experiments where the relative mobility of the labelled protein was compared to the mobilities of labelled marker proteins run in the same electrophoresis.

Incorporation of [^{3}H]felodipine into the 62 kDa protein was seen in about equal amounts also in samples of vascular smooth muscle proteins to which Ca^{2+} was not added prior to photolysis, 1214 ± 916 with a range from 245 to 3013 in presence of EGTA compared to 1142 ± 983 with a range from 346 to 3567 for Ca^{2+} added (mean ± SD, N = 11). In another set of experiments, labelling was performed with vascular smooth muscle proteins prepared as in Materials and Methods except that the preparation of the proteins was carried out in the presence of Ca^{2+}, which was added initially to the HEPES buffer in a concentration of 2 mM (EGTA not added). This difference in the Ca^{2+} concentration during preparation did not seem to influence the incorporation of radioactivity during the subsequent labelling experiments since it was seen also in protein solutions prepared in presence of Ca^2, 1202 ± 585 with a range from 440 to 1975 (mean ± SD, N = 6).

Irradiation of soluble proteins from vascular smooth muscle in the presence of [^{14}C]H 152/37, i.e. the corresponding oxidized pyridine analogue of felodipine, in a concentration of up to 50 μM did not result in any detectable labelling other than normal background (data not shown).

Irradiation of [^{3}H]felodipine and soluble proteins from vascular smooth muscle (prepared with EGTA and Ca^{2+} added prior to photolysis) at 254, 278 and >410 nm did not result in any detectable incorporation as seen when photolysing at 360 nm. Deoxygenation of the sample solutions with argon markedly enhanced the labelling of the 62 kDa protein at 360 nm (Fig. 2). The increased radioactivity in slice 19, 20 in this experiment was seen in a few experiments and may correspond to a protein labelled by the azidoanalogue of felodipine [7]. However, this labelling was not consistently seen and was, when present, always minor compared to the incorporation into the 62 kDa protein. Incorporation of [^{3}H]felodipine was increased after deoxygenation to values higher than ever seen when photolyzing samples exposed to air. We were not able to detect any incorporation above background to proteins of molecular weights between 90 and 45 kDa, when using for example 278 and >410 nm even after deoxygenation. Values for background were also increased at 360 nm after argon, but this was not the case in argon treated samples at the other wavelengths tested. These experiments were only performed twice at each wavelength but the successful labelling at 360 nm served as control.

As seen in Fig. 3, incorporation of radioactivity could not be found in gel slices with proteins ranging from 90 to 45 kDa (slices 11 to 20) when soluble proteins from liver, cardiac and skeletal muscle were UV irradiated at 360 nm in the presence of

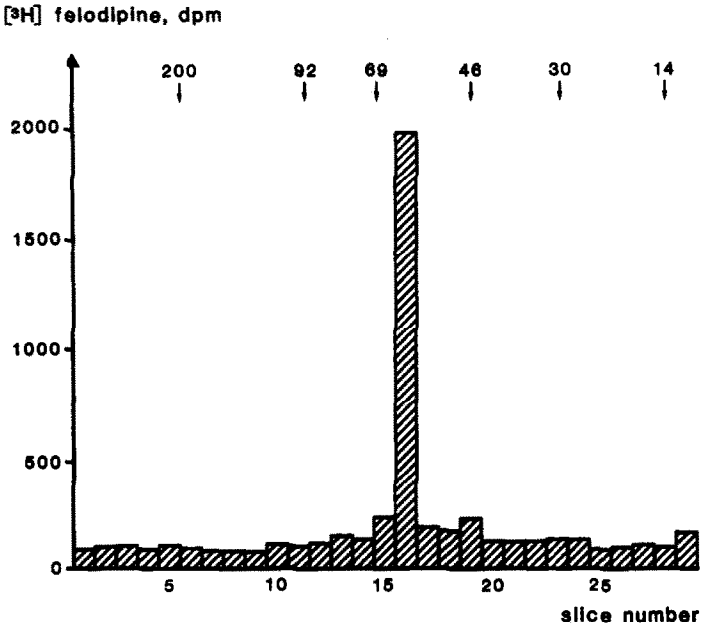

Fig. 1. Photoaffinity labelling of soluble proteins prepared from mesenteric vascular smooth muscle. The soluble proteins were irradiated with [^{3}H]felodipine as described in Materials and Methods. After SDS–PAGE, gels were sliced and counted for radioactivity. Arrows depict the location of molecular weight standards (in kDa).

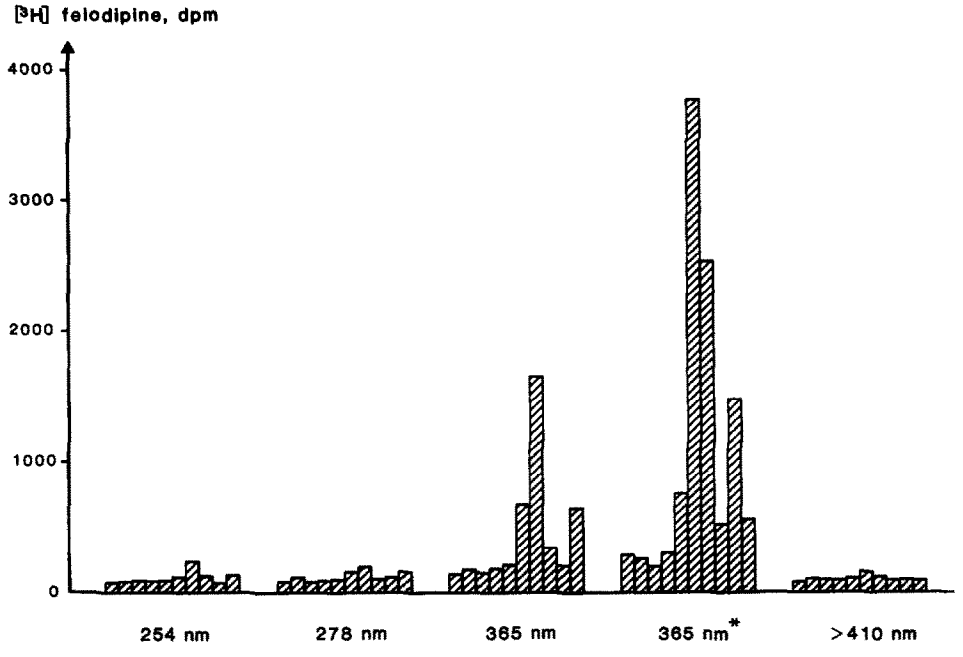

Fig. 2. Soluble proteins from mesenteric vascular smooth muscle were irradiated at 254, 278, 360 and above 410 nm in the presence of [^{3}H]felodipine. After SDS–PAGE, the gels were sliced and the radioactivity was measured in slices 11 to 20, which corresponded to molecular weights between approximately 100 and 45 kDa. *Indicates that the sample was deoxygenated with argon for 1 hr before irradiation.

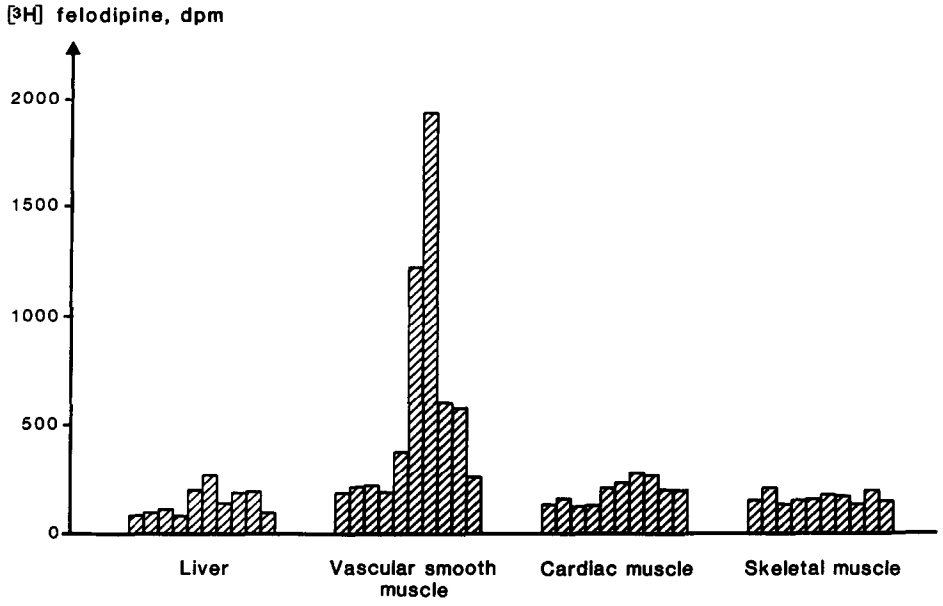

Fig. 3. Irradiation of soluble proteins prepared from different tissues in the presence of [^{3}H]felodipine as described in Materials and Methods. After SDS–PAGE, gels were sliced and the radioactivity was measured in slices 11 to 20, which corresponded to molecular weights between approximately 90 and 45 kDa.

[^{3}H]felodipine. However, photoaffinity labelling of the 62 kDa protein in vascular smooth muscle was present as in previous experiments. Labelling of vascular smooth muscle proteins were performed as control in each experiment, 1460 ± 640 (mean ± SD) with a range from 1026 to 2573, and five experiments with each of the other tissues were made.

DISCUSSION

The photosensitivity of 1,4-dihydropyridines is well known [10, 11]. On UV irradiation, it is possible that felodipine forms a radical intermediate which then can convert to the corresponding pyridine, H 152/37. If this occurs when felodipine is bound tightly to a functional protein responsible for its biological effect, the radical formed may bind covalently to this protein. Due to the expected short lifespan of such a radical intermediate, loose unspecific binding or diffusion and binding to other proteins should not be expected to result in any large extent of covalent bond formation. Experiments based on this assumption could thus provide a means of identifying the dihydropyridine binding site. When UV-irradiated, [^{3}H]felodipine was found to label a protein with an apparent molecular weight of 62 kDa in the soluble fraction prepared from vascular smooth muscle, indicating that felodipine is specially coordinated with this protein structure [7].

An alternative explanation for the observed labelling could be that there is a photosensitive protein that upon activation binds covalently to felodipine. The dihydropyridine moiety in the felodipine structure has an absorption mixture at 363 nm corresponding to the radiation peak at 366 nm of the UV-lamp. Since the UV-lamp used in the photoaffinity binding experiments had a rather broad spectrum, photoactivation of other structures was also possible. However, the fact that no labelling was observed when irradiating at 254, 278 and >410 nm suggests that felodipine was the activated molecule.

H 152/37 is the oxidized and pharmacologically inactive analogue of felodipine. The negative result obtained upon UV irradiation with [^{14}C]H 152/37 would support the theory of radical fixation since the pyridine compound is not likely to form a radical when UV-activated. A radical fixation could also explain the fact that the deoxygenated samples showed higher incorporation values, since oxygen could act as a scavenger of radicals. The argon treatment would therefore allow more activated felodipine molecules to react with the protein instead of with oxygen.

In another set of experiments, we tested whether this 62 kDa protein was present also in soluble fractions from liver and other muscular tissues where felodipine is believed to be without pharmacological effect in therapeutically relevant doses. Soluble protein fractions, prepared from cardiac, skeletal and vascular smooth muscle and liver, were UV irradiated in the presence of [^{3}H]felodipine.

Electrophoresis of the soluble protein fractions from all these tissues showed heavily stained protein bands in the 62 kDa range but [^{3}H]felodipine was only covalently incorporated into the vascular smooth muscle protein.

Although present in the soluble fraction of vascular smooth muscle cells, the 62 kDa protein labelled with [^{3}H]felodipine could be membrane associated in a Ca^{2+}-dependent manner but released

during the preparation [12]. However, the photoaffinity labelling experiments with soluble proteins prepared in the presence and absence of Ca^{2+} showed incorporation of [^{3}H]felodipine in both preparations. These results suggest that the 62 kDa protein is soluble also *in vivo* but the true localization of this protein remains unknown. The association of felodipine and the 62 kDa protein did not seem to be dependent on whether Ca^{2+} was present or not in the solution. The photoaffinity labelling of the vascular smooth muscle protein by [^{3}H]felodipine was of the same magnitude when performed in the presence of EGTA or when Ca^{2+} was added. Although the actual binding of felodipine to the protein does not seem to require Ca^{2+}, nothing is known about the possible involvement of this protein in Ca^{2+} dependent processes *in vivo*.

In conclusion, on UV irradiation, felodipine proved to be a photoaffinity ligand. The protein labelled seems to be present mainly in soluble fractions from vascular smooth muscle. The special coordination of felodipine to this protein, which is suggested for successful labelling, and the fact that no incorporation was found in proteins from the other tissues, indicates a possible functional importance of this 62 kDa protein. Use of felodipine as a photoaffinity ligand could thus prove to be of value for eludicating the molecular mechanism of action of antihypertensive dihydropyridines.

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