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In Vivo Inhibition of Dipeptidyl Peptidase IV Activity by Pro-Pro-diphenyl-phosphonate (Prodipine)

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ABSTRACT. Dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5), also known as CD26, is a membrane-bound serine protease that cleaves off aminoterminal dipeptides from peptides with a penultimate proline (or, at a much slower rate, a penultimate alanine). Recently, we synthesized and characterized a number of dipeptide-derived diphenylphosphonates. Out of the resulting series of slow-binding irreversible inhibitors of DPP IV, diphenyl 1-(*S*)-prolylpyrrolidine-2(*R,S*)-phosphonate hydrochloride (Pro-Pro-diphenylphosphonate or Prodipine) was selected for further study. We investigated the *in vivo* applicability of Prodipine. Male rabbits weighing 3–4 kg received a single intravenous injection with 10 mg Prodipine or saline. After 1 hr, plasma DPP IV activity had decreased to less than 20% of the preinjection value and remained unchanged during a 24-hr observation period. In a next step, we aimed to study (i) the dose dependency and (ii) the duration of the effect after a single intravenous dose of Prodipine. A profound and long-lasting inhibition of plasma DPP IV activity was observed in the treated animals (1, 5 or 10 mg). It took 5 to 8 days to reach half of the pretreatment DPP IV activity and generally more than 20 days for a complete recovery. Systemic treatment with Prodipine not only led to inhibition of plasma DPP IV activity but also decreased tissue DPP IV activity in circulating mononuclear cells, kidney cortex, thymus, spleen, lung, and liver. No differences in activities of the related peptidases aminopeptidase P (APP, EC 3.4.11.9), prolyl oligopeptidase (PO, EC 3.4.21.26), or aminopeptidase M (mAAP, EC 3.4.11.2) were detected between Prodipine-treated and control rabbits. The *in vivo* applicability of this chemically stable, irreversible inhibitor of DPP IV opens new possibilities, not only to further unravel the biological functions of this intriguing ectopeptidase, but also to explore this enzyme as a new target in various fields of pharmacological research. BIOCHEM PHARMACOL **54**;1:173–179, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. dipeptidyl peptidase IV; activation antigen cluster CD26; proteinase inhibitor; phosphonate; *in vivo*; proline

Dipeptidyl peptidase IV (DPP IV,‖ EC 3.4.14.5) is an ectoenzyme that occurs as an integral type II membrane protein on the cell surface [1]. Dipeptidyl peptidase IV is unique in combining a serine protease type mechanism with exopeptidase activity [2]. This ectoenzyme is a highly specific aminopeptidase, cleaving off dipeptides from the aminoterminus of peptides with a proline, hydroxyproline, or alanine at the penultimate position. By far the highest efficiency is observed with a proline residue [2]. The biological significance of proline motifs and proline specific enzymes was reviewed recently [3, 4]. DPP IV is present on a variety of epithelial and endothelial cells and is also found in body fluids (plasma, urine, and seminal fluid) [5]. Its expression is tightly regulated in different tissues and hematopoietic cells and strongly depends on the differentiation and activation status. In the hematopoietic system, DPP IV was identified as the activation antigen CD26 ([6], reviewed in [7]), and cells with a high surface density of CD26 have been shown to be responsible for the proliferation in response to recall antigen *in vitro* [8]. On the T cell surface, DPP IV/CD26 functions as adenosine deaminase binding protein [9, 10]. Triggering of CD26 by monoclonal antibodies provides a costimulatory signal for T cell receptor/CD3-mediated T cell activation [11–13]. The involvement of the DPP IV enzymatic activity in T cell activation was studied by different experimental approaches, and although several *in vitro* studies provide substantial evidence for a role of the catalytic activity [14–18], this issue remains a matter of debate [19–21]. More recently, low-density levels of DPP IV/CD26 have been detected on other hematopoietic cells, especially NK cells, B cells and

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‖ *Abbreviations:* APP, aminopeptidase P; DPP IV, dipeptidyl peptidase IV; mAAP, aminopeptidase M; MNC, peripheral blood mononuclear cells; PMN, peripheral blood polymorphonuclear cells; PO, prolyl oligopeptidase; and Pro-Pro-diphenylphosphonate (Prodipine), diphenyl 1-(*S*)-prolylpyrrolidine-2(*R,S*)-phosphonate hydrochloride.

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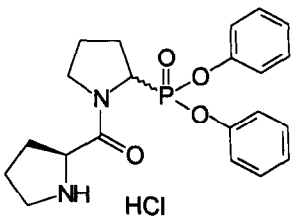

FIG. 1. Chemical structure of Prodipine, diphenyl 1-(S)-prolylpyrrolidine-2(R,S)-phosphonate hydrochloride.

myeloid cells [22–24]. As in T cells, expression increases upon activation of these cells. The unique proteolytic specificity points to a participation of this enzyme in peptide metabolism [3, 4]. Hydrolysis by DPP IV has already been proven for, among others, substance P [25, 26], neuropeptide Y [27], peptide YY [27], gastric inhibitory polypeptide [28], growth hormone releasing hormone [29], and glucose-dependent insulinotropic polypeptide [30].

Moreover, DPP IV is essential for the intestinal and renal transport of proline-containing peptides [31, 32]. The ability of this molecule to interact with proteins of the extracellular matrix might be important during cell migration, including infiltration processes [33–36]. Notwithstanding the rapidly increasing number of studies in the field of DPP IV/CD26 [37], we are far from a thorough understanding of the *in vivo* role(s) of this intriguing enzyme. Potent and specific biocompatible inhibitors have proved to serve as useful tools during functional studies of other enzymes.

Some chemical classes of DPP IV inhibitors have been synthesized and evaluated [14–16, 38–43], but most suffer from significant toxicity, which prohibits their introduction into biological systems. As far as we know, only the dipeptide prolyl boronic acid derivatives have been used *in vivo* up to now [44]. Although very potent and specific, their instability in aqueous solution at neutral pH limits their use. Diphenylphosphonate-based inhibitors of DPP IV have been synthesized independently by different groups of investigators [41, 42, 45]. Recently, we synthesized, chemically characterized, and evaluated a series of diphenylphosphonate derivatives (varying in their N-terminal residue) as slow-binding, specific inhibitors of DPP IV [43, 45]. One of them, diphenyl 1(S)-prolylpyrrolidine-2(R,S)-phosphonate hydrochloride (pro-pro-diphenylphosphonate) (Fig. 1), was given the trivial name Prodipine and selected for further study because of its efficiency and stability. When stored at 4°C, Prodipine remains fully active in aqueous solution for more than 24 hr (A. M. Lambeir *et al.*, unpublished data). At 37°C, the *in vitro* half-life of Prodipine in aqueous solution (pH 8.3) is approximately the same as in human citrated plasma (ca. 5 hr) [43]. In the present study, we report on the *in vivo* applicability and specificity of a dipeptide-diphenylphosphonate derivative as specific inhibitor for DPP IV.

MATERIALS AND METHODS

Drugs

Prodipine was synthesized as reported [45]. Solutions were freshly prepared in sterile saline at concentrations of 2 mg/mL.

Animals and Treatment

Male New Zealand white rabbits (3–4 kg) were purchased from the Rijksstation voor Kleinveeteelt, Merelbeke, Belgium. The animals (n = 20) were housed in individual cages and allowed to adjust to their new environment for at least 7 days. They received a standard commercial diet and water *ad lib.* A single slow intravenous (i.v.) bolus injection was given in the marginal ear vein. Blood was sampled from the central ear artery. Samples for haematological analyses were delivered to the laboratory within 1 hr of collection and were assayed promptly. After clotting, samples for biochemical analyses were centrifuged (3000 × g, 10 min), and the resulting sera were stored at −80°C until assayed.

EXPERIMENT 1 (PLASMA DPP IV ACTIVITY, 24-HR STUDY). The animals (n = 1 for each condition) received a single i.v. injection of 10 mg Prodipine or saline. Blood samples were taken 5 min before injection and 30 min, 1 hr, 2 hr, and 24 hr after the treatment.

EXPERIMENT 2 (PLASMA DPP IV ACTIVITY UPON DIFFERENT DOSES, 40-DAY STUDY). The rabbits (n = 1 for each condition) received a single i.v. bolus injection of saline or were treated with 1, 5, or 10 mg Prodipine (also in a single i.v. bolus) at time 0. The animals were kept under observation for 40 days. At several time points (−5 min, 4 hr, and at days 1, 2, 3, 6, 12, 21, and 40), blood samples were taken and body weight determined. Rabbits were killed with pentobarbital after termination of the observation period.

EXPERIMENT 3 (PLASMA DPP IV ACTIVITY, REPRODUCIBILITY OF EFFECT). The animals received a single i.v. bolus injection of saline or 5 mg Prodipine (n = 3 in each group). The animals were kept under observation. After 3, 7, 14, and 21 days, blood was taken and body weight determined.

EXPERIMENT 4 (ACTIVITY OF DPP IV AND RELATED PEPTIDASES IN TISSUES AND CELLS, 24-HR STUDY). The animals received a single i.v. bolus of 5 mg Prodipine (n = 4 in each group). Blood was taken prior to injection and 4 and 24 hr after administration of Prodipine. Thereafter, the animals were anesthetized (pentobarbital), and 40 mL blood was taken after cannulation of the carotid artery. After killing by pentobarbital overdosage, organs were taken immediately.

Enzyme Assays

Dipeptidyl peptidase IV activity was determined using the fluorogenic substrate Gly-Pro-4-methoxy-2-naphthylamide, as described previously [46]. Hydrolysis of L-Ala-4-methoxy-2-naphthylamide in 60 mM phosphate buffer pH 7.4 was chosen for the determination of aminopeptidase M activity. The intramolecularly quenched fluorogenic substrate Lys(2,4 dinitrophenyl)-Pro-Pro-NH-CH_2-CH_2-NH_2-aminobenzoyl · 2HCl was used to assay aminopeptidase P activity [5]. Prolyl oligopeptidase (PO) was measured with *N*-benzyloxycarbonyl-Gly-Pro-7-amino-4-methylcoumarin [47]. One unit of enzymatic activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of assay product per minute under the conditions used.

Dipeptidyl Peptidase IV Purification

Rabbit DPP IV was purified from kidney cortex. The homogenate was prepared in Tris 20 mM, pH 7.4 containing 1% Triton X-100 as solubilization buffer. Mixing was carried out on ice using a polytron aggregate mixer (Kinematica Ag, Littau, Switzerland). After centrifugation of the resulting suspensions (for 10 min at 2800 × *g*, Rotanta/TR centrifuge, Tuttlingen, Germany), pellets were discarded and the supernatants subjected to an ultracentrifugation at 120,000 × *g* for 60 min (Beckman Ultracentrifuge model L3-50, Beckman Instruments, Munich, Germany). The final supernatants were used immediately for enzyme purification using a recently reported method [48], including adenosine deaminase affinity chromatography [49]. The specific activity of the preparation obtained was 26,000 U/g.

Tissues

Immediately upon sacrifice, the organs were taken and rinsed with ice-cold PBS and kept on ice. Tissue samples (liver, kidney, thymus, lung, and spleen) for histologic examination were fixed immediately in 4% neutral formalin. Sections were evaluated after hematoxilin-Eosin staining. Within 2 hr, homogenates were prepared in cold PBS containing 1% Triton X-100 and 100 KIU/mL aprotinin (Trasylol, Bayer SA, Brussels, Belgium) as solubilization buffer. After mixing and centrifugation at low speed in the Rotanta centrifuge as described above, supernatants were transferred to Eppendorf tubes and centrifuged twice at 20,800 × *g* for 10 min. Centrifugations were carried out at 4°C. The final supernatants were kept at −80°C until enzyme assays and protein determinations.

Isolation of Rabbit Polymorphonuclear (PMN) and Mononuclear (MNC) White Cells From Peripheral Blood

Via cannulation of the central ear artery of the concious rabbits with a catheter (21G), 10 mL of citrated blood was taken at different time points of Experiment 4. At the end of the experiment and before the anaesthetized animals were killed, another 40 mL of citrated blood was collected via the cannulated carotid artery. The separation of leukocytes was performed at room temperature. After initial red cell sedimentation with hydroxyethylstarch (3% final concentration, Plasmasteril®, Fresenius A. G., Homburg, Germany) the supernatant was centrifuged to remove platelet-rich plasma. The leukocyte-rich pellet was resuspended and layered on a discontinuous Percoll/plasma gradient. The PMN-rich fraction was harvested followed by lysis of the remaining red cells. Differential leukocyte counting was performed by May-Grünwald Giemsa staining. The PMN preparation contained >95% PMNs. The MNC preparation contained >80% lymphocytes and approximately 5 to 15% monocytes.

Inhibition of Rabbit DPP IV In Vitro

The IC_{50} values of Prodipine towards rabbit DPP IV were determined as follows: rabbit plasma or equivalent amounts of purified rabbit DPP IV in Tris-buffered saline were incubated with appropriate inhibitor concentrations for 15 min at 37°C. After the addition of the substrate Gly-Pro-4-methoxy-2-naphthylamide to a final concentration of 1 mM, incubation was continued for another 10 min. The reaction was stopped by the addition of 500 μL 100 mM citrate pH 4.0. To assess the time dependency of the inhibitor binding in plasma, rabbit serum was incubated at 37°C with 10% v/v inhibitor dissolved in saline (final concentrations of Prodipine: 0, 50, 100, and 500 μM). After different time intervals, 5 μL thereof was added to 50 μL prewarmed Gly-Pro-4-methoxy-2-naphthylamide in Tris 50 mM pH8.3 (1 mM final concentration), and incubation at 37°C was continued for 20 min. The reaction was stopped by the addition of 500 μL 100 mM citrate pH 4.0.

Protein Determination

Protein concentrations were determined by the microassay procedure of Bradford [50], based on Coomassie Brilliant Blue G-250. Bovine serum albumin was used as standard. A correction for interfering substances was made using a blank.

Biochemical and Hematological Analyses

Biochemical assays (sodium, potassium, urea, creatinine, lactate dehydrogenase, and glutamyltransferase) were carried out with an Ektachem 400 TM Clinical analyzer (Eastman Kodak Co., Rochester, NY). Appropriate calibrators (Eastman Kodak Co.) were used for each assay. Values were compared with reference values for New Zealand white rabbits reported in [51]. Blood cells were counted on a Sysmex E 5000 (Dade, Brussels, Belgium).

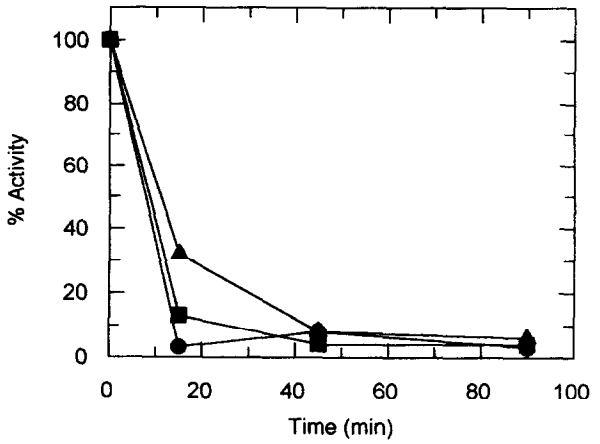

FIG. 2. *In vitro* time-dependency of rabbit plasma DPP IV inhibition. Rabbit serum was incubated at 37°C with different concentrations of Prodipine for several periods of time: 50 μM (triangle), 100 μM (square), and 500 μM (circle). Afterwards, the sample was diluted into Gly-Pro-4-methoxy-2naphthylamide (1 mM) and residual activity was measured for 20 min. DPP IV activities are expressed as percentage relative to a control sample without Prodipine.

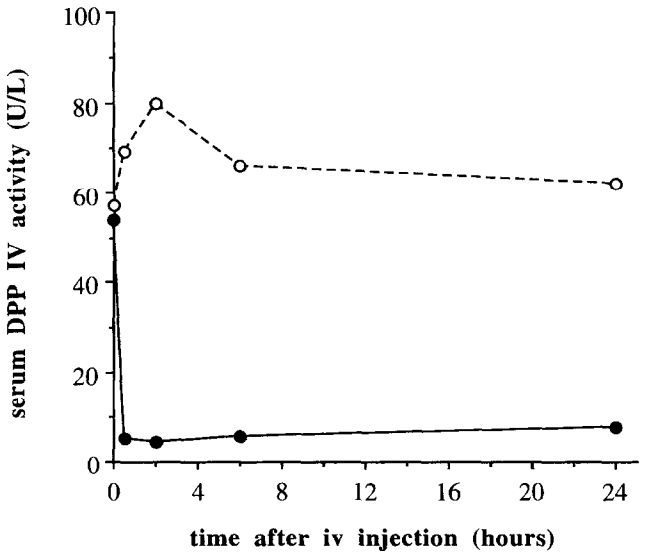

FIG. 3. *In vivo* effects of Prodipine. Serum DPP IV activity (U/L) up to 24 hr after a single i.v. bolus injection of 10 mg Prodipine (solid circles) or vehicle alone (open circles).

Statistical Analyses

The SPSS for Windows package (SPSS, Chicago, IL) was used for the analysis. A probability of error ≤0.05 was selected as the criterion for statistical significance. Serum and tissue DPP IV activities in vehicle- and Prodipine-treated animals were compared using Student's unpaired *t*-test. Equalities of variances were checked by Levene's test. When variances were not equal, logarithmically transformed values were analyzed (this was only the case for DPP IV kidney-specific activities). To compare DPP IV activity in mononuclear cells before and 4 hr after Prodipine treatment, Student's paired *t*-test was used.

RESULTS AND DISCUSSION

In this study we investigated the *in vivo* inhibition of DPP IV activity by Prodipine, a diphenyl-phosphonate derivative (Fig. 1) selected from a group of slow-binding irreversible inhibitors of this enzyme that we characterized recently [43]. To estimate the dose of Prodipine required for *in vivo* inhibition of DPP IV activity in rabbits, we measured *in vitro* inhibition of rabbit DPP IV (both purified from kidney cortex and the plasma enzyme in its natural matrix) by Prodipine. The IC_{50} values of Prodipine for purified and plasma DPP IV from the rabbit were 4.5 μM and 30 μM, respectively. The time course of *in vitro* inhibition of rabbit plasma DPP IV activity by different concentrations of Prodipine is given in Fig. 2. In a first *in vivo* experiment (Experiment 1), we observed a rapid and profound inhibition (>80%) of plasma DPP IV activity upon a single i.v. bolus injection of 10 mg Prodipine, which is consistent with *in vitro* results, taking into account a circulating volume of approximately 250 mL. Strikingly, the inhibition, which was already observed after 30 min, remained maximal during the entire 24-hr observation period (Fig. 3).

During a following set of experiments (Experiment 2), we aimed to study (i) the dose dependency, and (ii) the duration of the observed effect after a single i.v. dose of Prodipine. At the same time, hematological, biochemical, and histologic parameters were determined in order to roughly assess possible toxic side effects of the product. A profound and remarkably prolonged inhibition of plasma DPP IV activity was observed in treated animals. It took 5

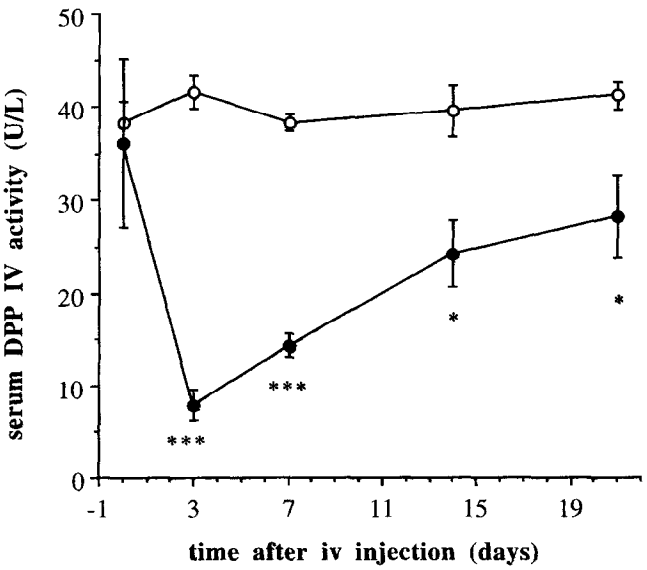

FIG. 4. Effect of a single 5 mg i.v. bolus injection of Prodipine on serum DPP IV. DPP IV activities (expressed in U/L) in Prodipine-treated (solid circles) and vehicle-treated (open circles) animals (n = 3 for each group). Data are shown as means ± SEM. ***P ≤ 0.001 and *P ≤ 0.05 (Student's unpaired *t*-test).

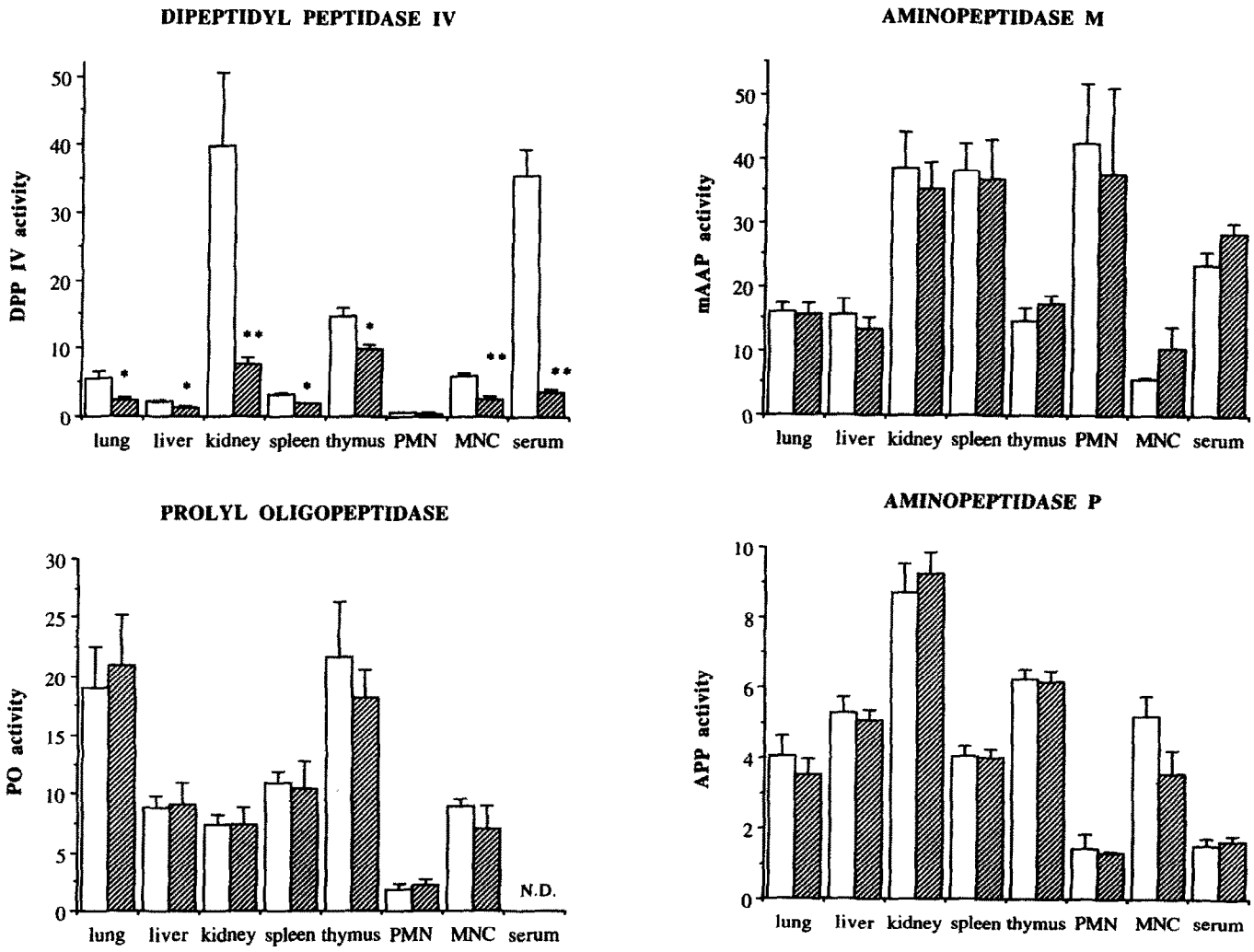

FIG. 5. Enzyme activities in tissues, cells and serum 24 hr after a single i.v. bolus injection of 5 mg Prodipine (hatched bars) or vehicle alone (open bars). For all tissues and cells, enzyme activities are expressed as U/g protein and for serum results are given as U/L. Data are shown as means ± SEM. **P ≤ 0.002 and *P ≤ 0.05 (Student's unpaired *t*-test). MNC and PMN: mononuclear and polymorphonuclear cells from peripheral blood. N.D.: not done.

to 8 days to reach half of the pretreatment DPP IV activity and generally more than 20 days for a complete recovery. *In vitro*, the half-life of Prodipine in a plasma matrix was approximately 5 hr. The long-lasting inhibition of plasma DPP IV activity might reflect both the *in vivo* stability of the inhibitor/enzyme complex and the rather slow recovery of plasma enzyme levels. The high *in vivo* stability of the DPP IV/Prodipine complex in comparison to the DPP IV/ProboroPro complex, together with the chemical stability of Prodipine in aqueous solution, constitutes an important improvement for future *in vivo* experiments on DPP IV. For doses ranging from 1 to 10 mg Prodipine, no dose–response relationship was found. At the first time point, 4 hr after Prodipine administration, as well as later during follow-up, the percentages of remaining DPP IV activity in serum were alike for the doses tested. During the entire 40-day observation period, no differences in general appearance, food intake, or body weight were observed between the groups. The hematological and biochemical parameters were not different in treated and control animals, nor could we observe clear shifts of values within individual animals (data not shown). Histologic examination of thymus, liver, spleen, lung, and kidney did not reveal any pathological alterations in the treated animals.

The time course of normalization of DPP IV activity in plasma upon a single i.v. bolus injection of 5 mg Prodipine proved to be very analogous for all individuals (Experiment 3). Figure 4 shows the DPP IV serum activities in control and Prodipine-treated animals. The DPP IV plasma levels were significantly lower compared with the control animals up to day 21.

DPP IV is known to be widely distributed in mammalian cells and tissues. To allow a correct interpretation of the pharmacological effects upon systemic administration of Prodipine, we analyzed the activity of DPP IV and other peptidases in various cells and tissues 24 hr after a single dose of Prodipine (Experiment 4, Fig. 5). Compared to control animals, DPP IV specific activity was significantly

lower in the kidney, liver, thymus, spleen, and circulating mononuclear cells. These tissues and cells bear DPP IV as an ectoenzyme on the plasma membrane, allowing Prodipine to interact with the active center without having to penetrate into the cell. For all tissues tested, *in vitro* addition of Prodipine (10 mM) was able to block >90% of the measured enzymatic activity, which points to the specificity of the assay. Our observation that systemic treatment with Prodipine not only leads to an inhibition of plasma DPP IV but also inhibits tissue DPP IV with a different degree of local efficiency corroborates similar findings in the plasma and tissue angiotensin-converting enzyme (ACE, EC 3.4.15.1) system. Using the well-studied and very potent inhibitors enalapril, ramipril and perindopril, different degrees of inhibition were observed [52].

DPP IV activity in mononuclear cells was determined immediately before Prodipine administration and 4 and 24 hr thereafter. Four hours after treatment, the specific DPP IV activity in mononuclear cells of treated animals was decreased to 60% of the initial value ($P = 0.011$). In the peripheral blood mononuclear cells, DPP IV activity remained at that level during 24 hr.

The *in vivo* specificity of Prodipine was verified by parallel determination of specific activities of the proline specific peptidases prolyl oligopeptidase and aminopeptidase P (APP), as well as the membrane alanyl aminopeptidase, better known as aminopeptidase M (mAAP). The results, shown in Fig. 5, demonstrate the *in vivo* specificity of Prodipine towards DPP IV. In plasma as well, the activities of the other enzymes tested remained unaltered upon Prodipine treatment (Fig. 5).

In conclusion, this study demonstrates the specific and long-lasting inhibition of plasma DPP IV activity upon a single dose of Prodipine in rabbits. Our findings with this irreversible inhibitor illustrate the slow recovery of DPP IV in plasma. In addition, we showed that Prodipine not only blocks plasma DPP IV activity but also inhibits DPP IV in circulating lymphocytes and peripheral tissues. Upon biochemical, haematological and histologic examination, no adverse effects were seen in healthy rabbits after a single dose of Prodipine (1–10 mg). The *in vivo* applicability of this class of chemically stable, irreversible inhibitors of DPP IV opens new possibilities, not only to further unravel the biological functions of this intriguing ectopeptidase, but also to explore this enzyme as a new target in various fields of pharmacological research.

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