



Cyclosporine A Decreases the Protein Level of the Calcium-Binding Protein Calbindin-D 28kDa in Rat Kidney

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ABSTRACT. Despite the widespread use of cyclosporine A (CsA), its mechanism of action and side effects are not yet completely understood. There exists a large body of evidence suggesting that disturbance of calcium homeostasis is a critical step in the cascade of cellular and molecular events induced by the drug. As recently shown in our laboratory by two-dimensional protein gel electrophoresis (2-DE) analysis of kidney homogenates, CsA induced numerous changes in several kidney proteins. One kidney protein in particular was shown to be strongly down-regulated by the drug. In this work we report the identification of the strongly decreased kidney protein as calbindin-D 28kDa, a vitamin D-dependent calcium-binding protein associated with calcium handling by cells. The assignment of the down-regulated protein spot is based on its internal amino acid sequence analysis and its specific reaction with a monoclonal antibody raised against calbindin-D 28kDa. In kidney homogenates of male Wistar rats treated with 50 mg/kg/d CsA for up to 28 days, calbindin levels were measured by ELISA and were shown to be continuously decreased with prolonged CsA treatment. To our knowledge, this is the first report describing the effect of CsA on kidney calbindin-D 28kDa protein levels. Further studies are needed to elucidate whether the CsA-mediated down-regulation of the calcium-binding protein calbindin-D 28kDa may be a critical factor for the renal adverse effects induced by this drug. *BIOCHEM PHARMACOL* 51;3: 253–258, 1996.

KEY WORDS. cyclosporine A; calcium-binding protein calbindin-D 28kDa; calcium transport; rat; kidney; two-dimensional electrophoresis

More than a decade ago it was shown that CsA^{||} exerts its immunosuppressive action by preventing T-cell proliferation via the inhibition of a Ca^{2+} -dependent event required for induction of (IL-2) transcription [1]. However, despite an immense increase in mechanistic understanding in recent years, the entire cascades of cellular and molecular events induced by CsA still remain speculative. It has been shown that CsA binds to an abundant 18-kDa protein named cyclophilin [2], and thereby inhibits its peptidyl prolyl isomerase activity [3]. The rotamase inhibitory activity of CsA was found to be a prerequisite, but not sufficient for potent immunosuppression [4, 5]. Calcineurin, a calcium/calmodulin-dependent serine-threonine phosphatase, was then identified as a target of the cyclophilin-CsA complex, and it was shown that its phosphatase activity was inhibited by the binding of the cyclo-

philin-drug complex [6]. The inactivation of calcineurin in turn led to the inhibition of the calcineurin-dependent activation of transcription factors, which ultimately regulate the transcription of the IL-2 gene [7, 8].

Many patients treated with CsA experience significant adverse effects [9]. A serious problem of nephrotoxicity has emerged, particularly in the treatment of allograft rejection [10]. The mechanisms by which CsA injures the kidney remain poorly understood. In experimental animals, the drug has been shown to cause acute renal vasoconstriction, followed by a decrease in glomerular filtration rate and renal blood flow [11, 12]. The mediators of renal vasoconstriction are not fully understood, but some of the vasoconstrictive effects might be calcium-dependent. Coadministration of calcium antagonists with CsA has been shown to prevent drug-induced impairment in renal blood flow [13], as well as post-transplant acute tubular necrosis [14]. Prolonged treatment with CsA appeared to result in microcalcification within or adjacent to tubular cells and, upon chronic administration of the drug, irreversible chronic renal failure with tubulointerstitial fibrosis and focal glomerulosclerosis has been described [15]. Whether these chronic effects are due to prolonged and persistent vasoconstriction and vascular damage is unclear.

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^{||} Abbreviations: CsA, cyclosporine A; 2-DE, two-dimensional electrophoresis; IL-2, interleukin-2; IEF, isoelectric focusing; SDS, sodium dodecyl sulfate; PAG, polyacrylamide gel; PBS, phosphate buffered saline; calcitriol, 1,25-dihydroxyvitamin D.

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Most recently, we reported the discovery of a kidney protein spot strongly down-regulated by CsA, as shown by 2-DE of male Wistar rat kidney homogenates [16]. In this work we describe the identification of this kidney protein spot as calbindin-D 28kDa, a vitamin D-dependent calcium-binding protein associated with calcium handling by cells [17], and we show its continuous decrease over prolonged treatment with CsA. To our knowledge, this is the first report that demonstrates the effect of CsA on kidney calbindin-D 28kDa protein levels.

MATERIALS AND METHODS

Animal Treatment Protocol and Sample Preparation

HanIbM: male Wistar rats (Biological Research Labs., Füllinsdorf, Switzerland), eight weeks of age and weighing 190–400 g, were used. Six groups of five rats were each treated with 50 mg/kg/day CsA by gavage (Sandimmun 100 mg/mL diluted 1:10 in olive oil) for 4, 10, or 28 days or with the vehicle solution (Sandimmun Placebo A diluted 1:10 in olive oil) for identical periods. The animals were killed with carbon dioxide gas on the day following the last treatment. Using a 1-mL Wheaton glass homogenizer, 150 mg of one end of a kidney was homogenized in eight volumes of 9M urea, 4% Nonidet P-40, 1% dithiothreitol (DTT), and 2% carrier ampholytes pH 8–10.5 (Pharmacia, Uppsala, Sweden). The homogenates were centrifuged at $420,000 \times g$ at 18°C for 12 min (TL100 ultracentrifuge, TLA 100.3 rotor, 100,000 rpm, Beckman Instruments, Palo Alto, CA, U.S.A.). The supernatant was removed divided into four aliquots and stored at –80°C until analysis.

Two-Dimensional Polyacrylamide Gel Electrophoresis

The standard gel running protocol was slightly modified to increase both the number of target spots and the amount of protein per spot, to collect enough material for amino acid sequencing.

Sample proteins were resolved using the 20 × 25 cm Iso-Dalt® 2-D system (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). First-dimensional isoelectric focusing (IEF) gels were prepared using pH 4–8 carrier ampholytes (BDH, Poole, U.K.). A 15 µL solubilized sample was applied to each gel, and the gels were run for approximately 34000 volt-hours using a progressively increasing voltage with a high-voltage programmable power supply. An Angelique™ computer-controlled gradient-casting system (Large Scale Biology Corporation, Rockville, MD, U.S.A.) was used to prepare the second-dimension sodium dodecyl sulfate (SDS) polyacrylamide gradient slab gels, in which the top 5% of each gel was 11%T acrylamide and the lower 95% varied linearly from 11% to 19%T. The acidic ends of three IEF gels (approximately 1/3 of the total length of each gel) were loaded side by side directly onto each slab gel without equilibration, and held in place by polyester fabric wedgies (Wedgies™, Large Scale Biology Cor-

poration, Rockville, MD, U.S.A.) to avoid the use of hot agarose; consequently, the number of IEF gels run was three times the number of slab gels (60 and 20, respectively). Second-dimensional slab gels were run overnight at 160 volts in cooled Dalt tanks (10°C) with buffer circulation.

Following SDS-electrophoresis, slab gels were fixed for 24 hours and stained with colloidal Coomassie Blue G (Serva, Heidelberg, Germany) for 5 days. The target spot was precisely excised from each IEF for protein sequencing, resulting in 3 spots per slab gel.

Peptide Mapping and Microsequencing

Excised 2D-spots from Coomassie Blue stained slab gels were submitted to proteolytic digestion in polyacrylamide gel (PAG) according to the method of Rosenfeld *et al.* [18]. Briefly, the excised gel pieces were washed with 40 mL of water for 2 hours. To remove most of the staining, they were transferred to a mixture of 40% acetone, 10% triethylamine, and 5% acetic acid in water pH 6.4, and heavily shaken for 30 min (orbital shaker, 300 rpm). They were then washed twice for one hour in 40 mL of water and incubated for 30 min in 50% acetonitrile. The solution was removed and the gel pieces were air dried for two hours. Digestion of protein spots was performed in an Eppendorf orbital mixer using a solution of 3 µg trypsin in 300 µL 100 mM Tris-HCl pH 8.2/10% acetonitrile. On each dried gel piece, 10 µL of the digestion solution was spotted, and pieces incubated for 20 hours at 37°C. The peptides were extracted twice for 30 min with 300 µL 60% acetonitrile/0.1% trifluoroacetic acid in the Eppendorf orbital mixer at 37°C. The pooled extracts were vacuum-dried, resolubilized in 20 µL 20% acetic acid, and stored at –20°C until analysis. 2D-spot derived tryptic peptides were diluted with 380 µL 0.1% trifluoroacetic acid and separated on a reverse phase column (C4 Vydac, 2.1 × 250 mm, Hesperia, CA, U.S.A.) using a 140B Solvent Delivery System (Perkin-Elmer, Foster City, CA, U.S.A.), and eluted with a gradient (7% to 70%) of acetonitrile in 0.1% trifluoroacetic acid. The column outlet was directly connected to a 1000S diode array detector (Perkin-Elmer), and peptide fractions were collected manually in Eppendorf tubes. Purified peptides were sequenced using a pulsed liquid model 477A sequencer equipped with an online 120 phenylthiohydantoin analyzer (Perkin-Elmer).

ELISA for Calbindin-D 28kDa

Calbindin-D 28kDa was quantified in kidney homogenates using ELISA techniques described by Miller and Norman [19]. Microtiter plate wells (MaxiSorp™, Nunc, Roskilde, Denmark) were each coated with 5 ng of rat recombinant calbindin-D 28kDa (SWant, Bellinzona, Switzerland) dissolved in 100 µL 50mM carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, 0.02% NaN₃, pH 9.6), and incubated overnight at 4°C. The plates were washed three times with 200 µL phosphate buffered saline (PBS), and reference calbindin or ho-

mogenized kidney tissue samples diluted out with PBS to parallel concentrations of the standard curve, and 100 μ L monoclonal anti-calbindin-D 28kDa (mouse asites fluid, Sigma, St. Louis, MO, U.S.A.) diluted 1:40,000 with PBS was added in each well. The setting was incubated overnight at 4°C. After washing with PBS, the wells were incubated overnight at 4°C with 100 μ L of an alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Sigma) diluted 1:1,000 with PBS. After washing, 100 μ L of the substrate p-nitrophenylphosphate dissolved in diethanolamine buffer (1 mg/mL) was added, and the alkaline phosphatase reaction stopped after 30 min by adding 100 μ L of 3N NaOH. In each well, the absorbance at 405 nm was measured using a microplate reader (Molecular Devices, Menlo Park, CA, U.S.A.). Calbindin-D 28kDa protein levels in rat kidney samples were calculated from the standard curve obtained with concentrations of 2 to 128 ng of reference calbindin diluted in PBS and spiked with tissue homogenisation buffer to parallel the kidney samples.

The protein content of the kidney samples was measured by a modified Bradford assay described by Ramagali and Rodriguez [20].

RESULTS

Two-Dimensional Polyacrylamide Gel Electrophoresis

A videoprint of a 2-D kidney protein pattern containing the acidic ends of three IEF gels is shown in Fig. 1. The arrow points to the protein spot previously shown to be strongly down-regulated by CsA. These modified slab gels allowed us to rapidly collect quantities of the target protein sufficient for subsequent amino acid sequence analysis.

Peptide Mapping and Microsequencing

Sixty-five target spots were excised from the Coomassie Blue stained 2D-gels and submitted to proteolytic digestion as described in Materials and Methods. The resulting peptide pattern is shown in Fig. 2. Peptide KYDTDH?GFIE (aa108-aa118, peak 15; ? corresponds to the aa serine, not detectable in this analysis system) and LFDSNNDG (aa152-aa159, peak 17) were positively identified (100% identity) as part of the amino acid sequence of calbindin-D 28kDa. Sequencing of peptide

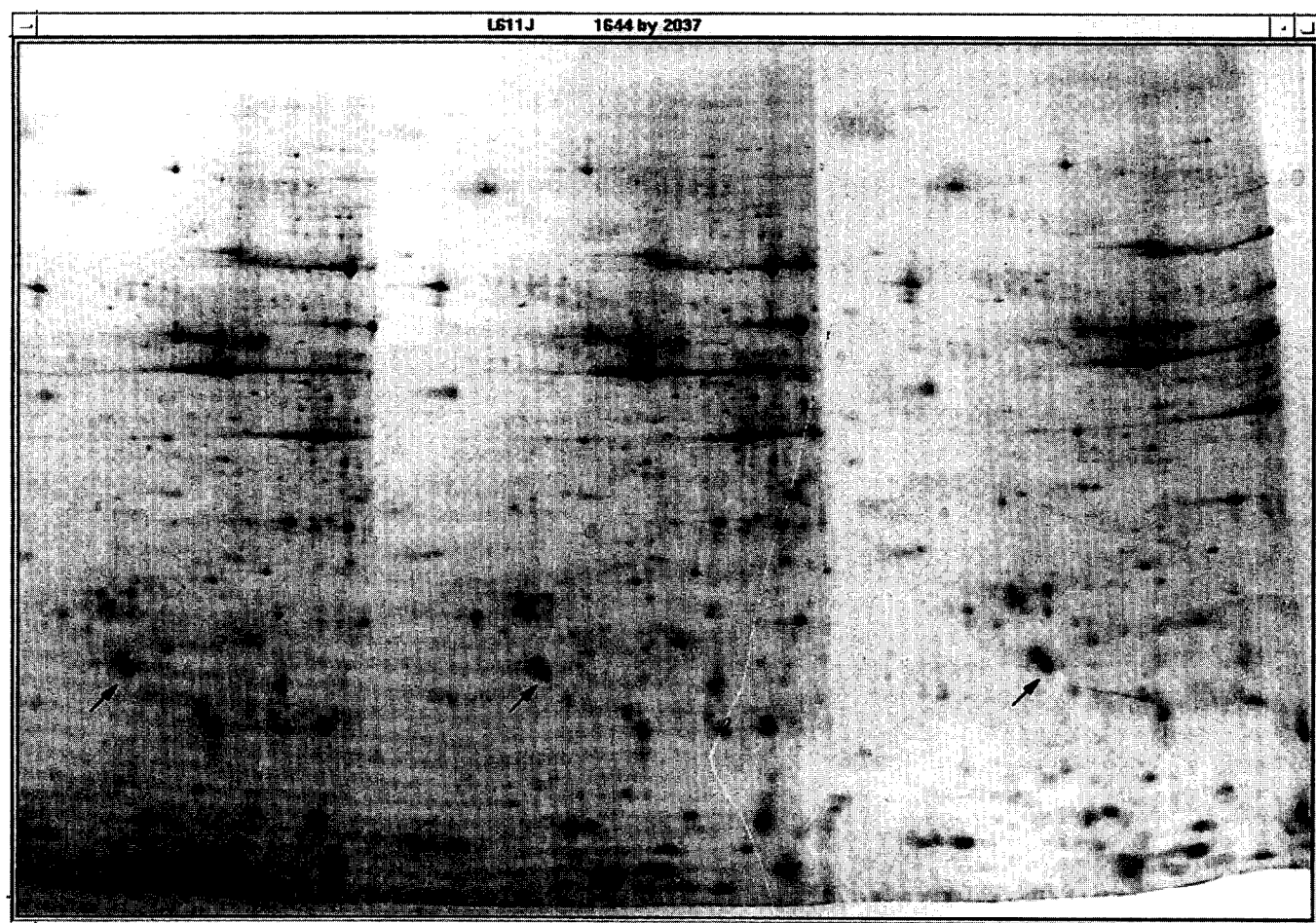


FIG. 1. A videoprint of 2-D rat kidney protein patterns produced for the collection of target spot 75. The arrows indicate the positions of protein spot 75, previously shown to be strongly decreased by CsA. In this 2-D pattern, the slab gel contains the acidic ends of three IEF gels.

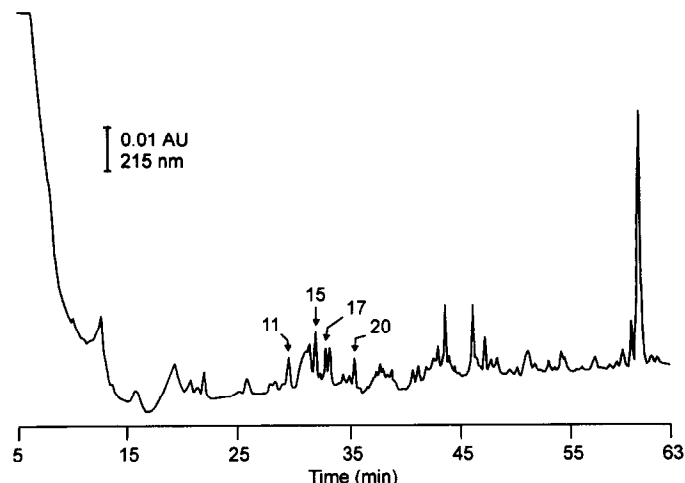


FIG. 2. Peptide pattern of 2D-spot 75 after proteolytic digestion in PAG with trypsin. Chromatography was performed on a C4 Vydac micro-bore (2.1×250 mm) column with a linearly increasing gradient of (A) 0.1% trifluoroacetic acid and (B) 70% acetonitrile in 0.1% trifluoroacetic acid with a flow rate of 300 $\mu\text{L}/\text{min}$ from 0 to 5 min (constant 10% B), 150 $\mu\text{L}/\text{min}$ from 5 to 65 min (to 70% B), 150 $\mu\text{L}/\text{min}$ from 65 to 66 min (to 100% B), and 150 $\mu\text{L}/\text{min}$ from 66 to 75 min (constant 100% B). Arrows indicate peptide peaks that were submitted to sequencing. The amino acid assignments of peptide peaks 15 and 17 showed 100% homology with the sequence of calbindin-D 28kDa. No valid sequence data was obtained from peak 11. Peptide peak 20 was identified as part of bovine trypsin, deriving from autodigestion of the enzyme.

peaks 15 and 17 was performed with an initial yield of 3 and 18 pmol, respectively. No valid sequence data was obtained from peak 11. Peptide peak 20 was identified as part of bovine trypsin, deriving from autodigestion of the enzyme.

Calbindin-D 28kDa Protein Levels in Kidneys of Control and CsA-Treated Rats

In the kidneys of CsA-treated rats, a time-dependent decrease in calbindin-D 28kDa levels was observed, as shown in Fig. 3. After four days of treatment calbindin protein levels were reduced by 20%, after ten days by more than 80%, and after 28 days the decrease was greater than 95% as compared to the respective controls. The decrease in calbindin was highly significant statistically ($P < 0.001$, Student's *t*-test) versus the corresponding controls after ten and 28 days of CsA treatment. A continuous treatment-related downward trend was evident at high statistical significance: Each treated group showed a $P < 0.001$ difference from the other groups. A slight but not significant decrease in calbindin levels was observed in the controls of the 28-day treatment group in comparison with the controls of the 10-day treatment group.

DISCUSSION

Since its discovery in 1972 [21], the mode of action of CsA has been the subject of a considerable number of studies in pre-clinical and clinical research. An essential step forward in the

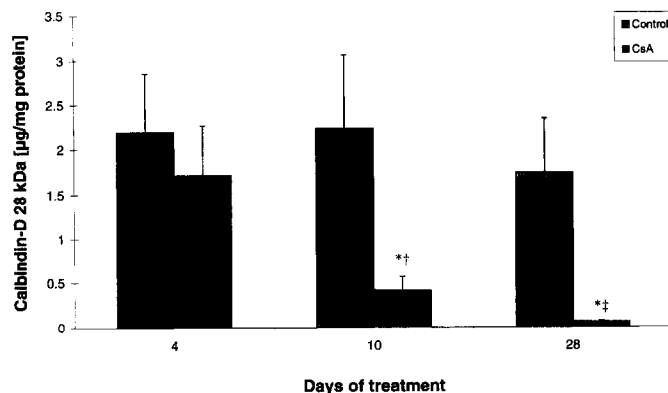


FIG. 3. Effect of CsA on rat kidney calbindin-D 28kDa protein levels as measured by ELISA. Rats were given CsA (50 mg/kg/day) by gavage for 4, 10, and 28 days. The bars represent the means \pm SEM of 5 rats. Key: significantly different ($P < 0.001$, Student's *t*-test); *treated vs corresponding controls; †treatment day 10 vs day 4; or ‡treatment day 28 vs day 10.

mechanistic understanding of the drug's action was the demonstration of its inhibitory effect on the calcium/calmodulin-dependent phosphatase calcineurin [6]. Calmodulin is known as one of the most important cytosolic receptor proteins for the second messenger calcium, and the calcium-calmodulin complex was shown to regulate a number of physiological processes [22]. Interestingly, the kidney protein calbindin-D 28kDa, which is heavily down-regulated by CsA as shown in this work, is likewise a cytosolic calcium-binding protein with characteristics of known calcium-sensitive proteins such as troponin C and calmodulin, including a conformational change in the protein with calcium-binding [23]. Little is known about the physiological role of calbindin. It has been postulated to function as a calcium transport molecule that facilitates the diffusion of calcium through the cell and serves as an intracellular calcium buffer, maintaining the ionized calcium below toxic levels during transcellular calcium transport [24]. The protein is found in many mammalian species and in various tissues, with highest concentrations in calcium-transporting tissues such as intestine, kidney, and placenta [25]. Several workers have shown that in the kidney the highest amounts of calbindin are localized in the distal tubule, which correlates with the role of the distal tubule as the site of calcium absorption [26, 27].

Recently CsA has been shown to increase the transport rate of calcium across liquid CH_2Cl_2 membranes, indicating a drug-induced disorder in calcium metabolism [28]. It needs to be investigated whether and how the calcium ionophore activities of CsA might be related to the drug-induced decrease in kidney calbindin-D 28kDa protein levels.

The expression of the kidney calbindin-D 28kDa is known to be induced by the hormonal and biologically active form of vitamin D, 1,25-dihydroxyvitamin D (calcitriol) [25]. The biological actions of calcitriol appear to be mediated by a signal transduction mechanism involving a cytosolic/nuclear receptor for calcitriol that modulates gene expression. To date, at least 28 tissues have been shown to possess the calcitriol receptor, including bone, kidney, liver, pancreas, skin, and thy-

mus [29]. The potent steroid hormone is known to regulate a variety of genes or gene products, among which are the biosynthesis of calcium-binding proteins and lymphokines [30]. In the immune system, calcitriol appears to act at the sites of inflammation to inhibit T-cell proliferation as well as IL-2 production, and to activate macrophage cytotoxicity [31]. Further investigations will be necessary to elucidate the relationship between the function of calcitriol on differentiation events in the immune system and its effect on mineral homeostasis. There seems to be good evidence that the discovery of the CsA-mediated down-regulation of kidney calbindin-D 28kDa protein levels is an exciting new piece of information supporting the effort to elucidate the molecular mechanisms of this fascinating drug.

The availability of a monoclonal anti-calbindin-D 28kDa antibody allowed rapid quantification of the calbindin-D 28kDa in whole kidney homogenates by ELISA. The specific reaction of the antibody with calbindin-D 28kDa was confirmed by blotting a 2-D gel onto a PVDF membrane followed by immunostaining with the anti-calbindin-D 28kDa antibody, which resulted in the staining of the corresponding calbindin-D 28kDa spot in the 2-D pattern (data not shown). Quantification of calbindin-D 28kDa in kidney homogenates of rats treated with CsA for different periods of time showed that the calcium-binding protein constantly decreased with prolonged treatment. Further studies will be needed to determine at precisely which time of treatment the down-regulation of the calcium-binding protein first becomes obvious, and how the decrease in this protein relates to the onset of histopathological changes observed in kidney. As calbindin acts in kidney tubule epithelial cells as a cytosolic facilitator of Ca^{2+} diffusion from the brush border membrane to the basolateral membrane, it could be rationalized that the CsA-induced corticomedullary mineralization found in kidney may be related to decreased calbindin levels. Although this is a compelling hypothesis, further studies are necessary to investigate the relationship between the CsA-induced decrease in kidney calbindin protein levels and the drug's adverse effects in kidney.

This work reports the identification of a rat kidney protein spot previously shown to be decreased in 2-D kidney protein patterns of CsA-treated male Wistar rats [16]. Rat kidney homogenates were applied to the 2-D system without prior cellular or subcellular fractionation, target protein spots were excised from a number of 2-D gels, and the pooled spots were subjected to amino acid sequence analysis. This approach should generally be valid for the identification of protein spots from 2-D gel patterns, provided the spots represent known proteins with published amino acid or DNA sequences. Undoubtedly, 2-D protein gel electrophoresis combined with amino acid sequence analysis carries an enormous potential to discover new drug targets at the molecular level. As the number of identified spots on the 2-D patterns continues to grow, the value of this tool for experimental pharmacology and toxicology will constantly increase.

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