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Protein kinase C isoenzymes in rat and human cardiovascular tissues

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1 We have compared the expression of protein kinase C (PKC) activity and immuno-detectable isoenzymes in cytosolic and membrane extracts of rat and human cardiovascular tissues (heart, kidney, aorta, saphenous vein). Experiments were performed in raw extracts and upon combined diethylaminoethylcellulose (DEAE) and phenylsepharose column chromatography.

2 PKC activity that bound to DEAE mostly eluted with 200 mM NaCl. DEAE-purified PKC from all tissues except rat kidney bound almost quantitatively to phenylsepharose and eluted with 0.5–0 M NaCl.

3 Immunoblots with an antibody against classical PKCs and the activator profile for phosphatidylserine, diolein and Ca^{2+} revealed that the PKC from rat kidney, which did not bind to phenylsepharose, was most probably due to a proteolytically-generated, constitutively active PKC which is not under the control of a regulatory subunit.

4 Studies in the reference tissue, rat brain, demonstrated that all PKC isoenzymes investigated (classical PKCs α, β, γ, new PKCs δ, ε, η, θ, and atypial PKCs ζ, λ, ι) have similar DEAE and phenylsepharose chromatography elution profiles. In the functional assay an inhibitor of all known PKC isoenzymes, bisindolylmaleimide, and a specific inhibitor of classical PKCs, Gö 6976, both inhibited PKC from rat brain completely and with high potency indicating that the functional assay preferentially detects classical PKC isoenzymes.

5 Each PKC isoenzyme had a tissue-specific expression profile which was similar in rat and man. The classical PKCα, the new PKCs δ and ε and all atypical PKCs were detectable in most tissues, whereas the PKCβ and PKCγ were not detected in any pheripheral tissue; PKCη and PKCθ were found in some tissues.

6 We conclude that combined DEAE and phenylsepharose chromatography is useful to enrich and detect PKC isoenzymes; no major species differences in tissues-specific expression patterns appear to exist between rat and man.

Keywords: Protein kinase C isoenzymes; brain; kidney; heart; aorta; vein

Introduction

Protein kinase C (PKC) is a family of closely related serine/threonine kinases. They play a crucial role in cellular signal transduction and can regulate cellular effector functions, growth and differentiation (Asaoka *et al*., 1992; Dekker & Parker, 1994). In the cardiovascular system PKC has been implicated e.g. in vasoconstriction (Bilder *et al*., 1990; Silver *et al*., 1992; Aburto *et al*., 1995), ischaemic preconditioning (Tsuchida *et al*., 1994; Baxter *et al*., 1995), and development of cardiovascular hypertrophy (Dunnmon *et al*., 1990; Sei *et al*., 1991). According to their structural homologies the members of the PKC family are divided into three groups (Nishizuka, 1988; 1992). The first group are the classical PKC (cPKC) isoforms, α, βI, βII and γ, which require phospholipids, Ca^{2+} and diacylglycerol or phorbolester for activation. cPKCs consist of five variable and four conserved regions. The functional domains are found in the conserved regions (C-regions). The C4 region contains the catalytical centrum, the C3 region contains the ATP binding site, the C2 region is responsible for Ca^{2+}-binding, and the C1 region, which contains two cysteine rich zinc-fingerlike regions, is responsible for diacylglycerol or phorbolester binding. The second group of new PKC (nPKC) isoforms (δ, ε, η and θ) are lacking the Ca^{2+}-binding C2 region, and therefore do not require Ca^{2+} for activation. The third group of PKC isoenzymes (ζ, λ and ι) are termed atypical PKCs (aPKC). They lack a C2 region and their C1 region contains only one zinc fingerlike region. PKCλ and PKCι may be species homologues. The aPKC isoenzymes do not require the presence of Ca^{2+} or phorbolesters for activation, but recent work has shown that aPKCs are targets of lipid second messengers such as ceramide, phosphatidic acid and 3′-phosphoinositides (Diaz-Meco *et al*., 1996). Moreover, PKC isoforms differ in their tissue distribution, their susceptibility to down-regulation upon phorbolester treatment, and possibly their substrate specificity (Nishizuka, 1992; Dekker & Parker, 1994). Therefore, it is likely that different PKC isoforms subserve distinct biological functions.

To understand which PKC isoenzymes are involved in which cardiovascular function, it is necessary to establish in which tissues and cell types they are preferentially expressed. PKC isoenzyme expression has been investigated either at the mRNA level by RNA protection assays (Seynaeve *et al*., 1994) or Northern blotting (Selbie *et al*., 1993; Akimoto *et al*., 1994) and at the protein level immunologically with PKC isoenzyme specific antibodies. While many studies have described the isoenzyme expression pattern in rat tissues (Kosaka *et al*., 1988; Qu *et al*., 1991; Wetsel *et al*., 1992; Selbie *et al*., 1993; Bogoyevitch *et al*., 1993; Church *et al*., 1993; Rybin & Steinberg, 1994; Clerk *et al*., 1995), little is known in man. Moreover, most studies have been performed with total protein extracts from tissues or cells (Wetsel *et al*., 1992; Bogoyevitch *et al*., 1993; Rybin & Steinberg, 1994). However, PKC isoforms of low abundance may be only poorly detectable in total protein extracts of some tissues (Wetsel *et al*., 1992; Bogoyevitch *et al*., 1993), and partial purification by column chromatography may increase sensitivity of detection (Bogoyevitch *et al*., 1993). Finally, all PKC isoforms are mainly found in the

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cytosol under resting conditions and translocate to the membrane fraction upon activation (Nishizuka, 1992) but few studies have assessed the cytosol/membrane distribution of PKC isoenzymes. Therefore, we have compared PKC isoenzyme expression patterns in cytosol and membrane fractions of rat and human cardiovascular tissues (heart, kidney, aorta, saphenous vein) in comparison with rat brain, which served as a well-investigated reference tissue. For this purpose we have used total protein extracts of the cytosolic and membrane fraction (Triton X-100 soluble fraction) and have further purified PKC isoenzymes of both fractions by combined diethylaminoethyl cellulose (DEAE) and phenylsepharose column chromatography. While DEAE and phenylsepharose column chromatography have previously been used to purify PKC from rat brain (House *et al*., 1987; Walton *et al*., 1987; Allen *et al*., 1994), it is not known whether this is similarly useful for all PKC isoforms.

Methods

Tissue preparation

Rat brain (cerebral cortex), heart, aorta, and kidney were obtained from adult male Wistar rats (Lippische Versuchstierzucht, Extertal, Germany). Human aortae were from patients undergoing heart transplantation (recipients). Human right atria and saphenous veins were from patients without apparent signs of heart failure undergoing aortocoronary bypass surgery. Human kidneys were from patients undergoing nephrectomy because of renal cancer; only tissue that was macroscopically free of tumours was used. All tissues were macroscopically cleared from adhering connective tissue, immediately frozen in liquid nitrogen, and stored at -80°C until analysis.

Tissues were prepared as previously described (Erdbrügger *et al*., 1995). Briefly, tissues (3 g each) were homogenized in $\approx$10 vol of buffer A (20 mM Tris, 2 mM EGTA, 1 mM DTT, 1 mM PMSF, 50 μg ml^{-1} soybean trypsin inhibitor, 10 μM pepstatin A, 10 μM leupeptin and 2 μg ml^{-1} aprotinin at pH 7.4) by use of a combination of an Ultra-Turrax (Jahnke & Kunkel, Staufen, Germany) and a Dounce homogenizer. The homogenates were centrifuged twice for 20 min at 50,000 *g* and the combined supernatants were considered as the cytosol extract; the washed pellet was considered as the membrane fraction. The membrane fraction was resuspended in buffer A containing 1% Triton X-100, incubated for 30 min at 4°C, and centrifuged twice again for 20 min at 50,000 *g*; the combined supernatants were used as the membrane extract.

Column chromatography

Extracts were placed on DEAE columns equilibrated with buffer A containing 0% (cytosolic extracts) or 1% Triton X-100 (membrane extracts). The columns were washed with buffer B (20 mM Tris, 1 mM EDTA, 1 mM DTT, 1 mM PMSF at pH 7.5), and then eluted stepwise with buffer B containing 200 mM and 400 mM NaCl. The 200 mM NaCl fraction from the DEAE column was adjusted to 1.5 M NaCl and applied to phenylsepharose columns equilibrated with buffer B supplemented with 1.5 M NaCl. Following washout of unbound protein with the same buffer, the columns were eluted stepwise with buffer B supplemented with 1.0, 0.5 or 0 M NaCl as described previously (House *et al*., 1987). The eluates were concentrated 4 fold by ultrafiltration with Centrisat I at 2500 *g* for 10–20 min.

PKC activity assay

The PKC assay was performed as previously described (Erdbrügger *et al*., 1995). Briefly, samples were diluted with buffer B supplemented with 0.5 mg ml^{-1} BSA. Thereafter, the extracts were incubated for 5 min at 30°C in a final volume of 50 μl containing unless otherwise indicated 20 mM Tris, 0.85 mM $CaCl_2$, 20 μM [^{32}P]-ATP (750 c.p.m. pmol^{-1}) phosphatidylserine and 10 μg ml^{-1} diolein. [S^{25}]-PKCα_{19-31} (RFARKGSLRQKNV; 25 μM) was used as a specific PKC substrate. When Ca^{2+} was omitted, 0.5 mM EDTA was present in the assay instead. The phosphorylated substrate was quantified by its binding to phosphocellulose paper (Whatman P81) as described previously (Walton *et al*., 1987). In this assay one unit is defined as the PKC activity which incorporates 1 pmol phosphate into the substrate per minute at 30°C.

Immunoblotting

Samples were boiled in Laemmli buffer at 95°C for 5 min and applied to 9% polyacrylamide gels. After gel electrophoresis (30 mA/gel, approximately 5 h, 4°C) the proteins were transferred to nitrocellulose (55 V, 12 h, 4°C). All the following steps were performed at room temperature. The membranes were blocked with Tris buffered saline (TBS: 20 mM Tris, 120 mM NaCl) containing 5% low fat milkpowder for 90 min and washed twice with TBS containing 0.05% Tween 20 (TTBS) for 10 min. Thereafter, the PKC antibodies (the monoclonal anti-PKCλ and anti-PKCι at 1 μg ml^{-1} all polyclonal antisera at 4 μg ml^{-1}) were incubated for 1 h in TBS containing 1% low fat milkpowder. The blots were washed twice with TTBS for 10 min. Detection of the primary antibodies was performed with [^{125}I]-protein A (polyclonal antisera) as described by Michel-Reher *et al*. (1993) or with the ECL system (monoclonal antibodies) according to the manufacturers instructions.

Materials

Standards of human recombinant PKCα, PKCβII, PKCγ, PKCδ, PKCε, PKCη, and PKCζ were from Calbiochem (San Diego, CA, U.S.A.). For Western blot detection the following polyclonal rabbit antisera raised against the indicated peptides sequences were obtained: antisera against PKCα (rat amino acid residues 313–326), PKCβ (rat, 313–329), PKCγ (rat, 306–318), PKCδ (rat, 662–673), PKCε (rat, 726–737), PKCζ (rat, 577–592) were from GIBCO BRL (Gaithersburgh, MD, U.S.A.), and antisera against PKCδ (human 657–676), PKCη (mouse, 669–683) and PKCθ (mouse, 656–671) were from Santa Cruz Biotech (Heidelberg, Germany). A rabbit polyclonal antiserum directed against a common domain of PKCα, PKCβ and PKCγ (human, 528–537) was also obtained from Santa Cruz Biotech. Monoclonal anti-PKCι (404–587) and anti-PKCλ (mouse, 397–558) antibodies were from Transduction Laboratories (Lexington, KT, U.S.A.).

ECL Western blotting development system was from Amersham (Buckinghamshire, U.K.). [^{125}I]-protein A (70–100 μCi μg^{-1}) and [γ-^{32}P]-ATP (3000 Ci mmol^{-1}) were from New England Nuclear (Bad Homburg, Germany). [S^{25}]-PKCα_{19-31} was from SERVA (Heidelberg, Germany). Centrisat I spin columns (10 kDa cut off) were from Sartorius (Göttingen, Germany). Phenylsepharose CL 4B was from Pharmacia Biotech. (Uppsala, Sweden), diethylaminoethyl cellulose (DEAE,DE-52) and phosphocellulose paper (P81) were from Whatman (Maidstone, U.K.). Phosphatidylserine, diolein, bovine serum albumin (BSA), dithiothreitol (DTT), aprotinin, leupeptin, phenylmethylsulphonylfluoride (PMSF) and soybean trypsin inhibitor were from Sigma (Deisenhofen, Germany). The PKC inhibitors, bisindolylmaleimide I (also known as GF 109203X or Gö 6850) and Gö 6976 (Martiny-Baron *et al*., 1993) were from Calbiochem. All other chemicals were purchased from Merck (Darmstadt, Germany).

Results

We have initially investigated whether PKC activity from all eight tissues is similarly enriched upon combined DEAE and phenylsepharose column chromatography. The main fraction

of the applied protein (60–90%) from extracts of all tissues did not bind to the DEAE columns, 10% or less was eluted with 400 mM NaCl and the remaining fraction eluted with 200 mM NaCl (data not shown). Membrane PKC activity from all tissues bound to DEAE columns, and in most tissues the major fraction of PKC activity was eluted with 200 mM NaCl (Table 1). Membrane PKC activity from human aorta and vein bound more tightly and was mainly eluted by 400 mM NaCl (Table 1). Cytosolic PKC activity from all tissues bound less tightly to DEAE columns than membrane PKC, and in some tissues (rat and human heart and kidney) a major fraction of cytosolic PKC activity did not bind to the DEAE columns (Table 1). Membrane PKC activity represented only a minor fraction of total cellular PKC activity (rat brain 16±2%, rat kidney 15±3%, rat heart 13±4%, rat aorta 24±5%, human kidney 30±8%, human heart 14±1%, human aorta 18±9%, human vein 7±2%; $n=4-5$) with the major fraction residing in the cytosolic extracts. Since our experiments have been performed on frozen samples, sample PKC degradation related to freeze-thawing cannot be excluded, but use of fresh samples was not possible for technical reasons; moreover, freeze-thawing related PKC degradation, if any, should be the same for all samples.

To assess the degree of purification by the DEAE column, we have determined specific PKC activities in each fraction (Table 1). As expected, specific activity was smallest for all tissues in the fraction which did not bind to the column. In most tissues (except for human aorta and vein) specific activity was highest in the 200 mM NaCl fraction. As expected, the highest specific activity of cytosolic and membrane PKC was found in rat brain (15.4 and 7.3 u mg^{-1} protein, respectively). Fairly high specific activities were also found in human kidney membrane extracts (6.5 u mg^{-1} protein) and in rat aorta cytosolic and membrane extracts (2.1 and 2.7 u mg^{-1} protein,

Table 1 Partial purification of protein kinase C (PKC) from rat and human tissues by DEAE column chromatography

		PKC activity (% of total)			*Specific PKC activity* (u mg^{-1} protein)		
		Not bound	*NaCl 200 mM*	*NaCl 400 mM*	*Not bound*	*NaCl 200 mM*	*NaCl 400 mM*
Rat brain	C	37±5	61±5	2±0	3.10±0.50	15.40±3.00	1.25±0.40
	M	20±3	78±3	2±1	0.18±0.04	7.30±1.90	0.85±0.30
Rat kidney	C	49±8	47±8	4±1	0.13±0.03	0.28±0.05	0.30±0.14
	M	0±0	86±8	14±8	0.00±0.00	0.28±0.18	0.13±0.05
Rat heart	C	68±4	29±3	3±0	0.28±0.02	0.43±0.05	0.45±0.09
	M	23±14	72±12	5±2	0.03±0.02	0.70±0.11	0.13±0.06
Rat aorta	C	11±3	79±4	10±5	0.16±0.10	2.19±1.02	0.68±0.45
	M	15±11	78±13	7±3	0.07±0.05	2.68±0.44	1.06±0.47
Human kidney	C	63±4	35±3	2±1	0.70±0.10	0.85±0.22	0.63±0.29
	M	1±1	95±1	4±1	0.00±0.00	6.50±1.70	0.58±0.14
Human heart	C	43±7	50±6	7±1	0.25±0.05	0.50±0.11	0.45±0.10
	M	33±9	56±8	11±1	0.06±0.02	0.60±0.10	0.38±0.06
Human aorta	C	24±14	34±11	42±17	0.01±0.01	0.02±0.01	0.16±0.07
	M	13±13	6±4	81±17	0.01±0.00	0.01±0.00	0.17±0.06
Human saphenous vein	C	3±2	36±6	62±8	0.02±0.01	0.19±0.05	0.55±0.17
	M	2±2	12±8	86±8	0.00±0.00	0.03±0.02	0.63±0.08

Cytosolic (C) and membrane (M) extract were applied to DE 52 columns and eluted stepwise with buffer containing NaCl 0 mM (not bound), 200 mM and 400 mM. The left side of the table shows the PKC activity as % of total PKC activity (sum of not bound, 200 mM and 400 mM NaCl fraction) taking into account the amount of protein eluted in the fractions and their specific activity (right side). The right side of the table shows the specific PKC activity in the different DEAE fractions. Data are mean±s.e.mean of 4–5 independent experiments.

Table 2 Purification of protein kinase C (PKC) from rat and human tissues by phenylsepharose column chromatography

		PKC activity (% of total)				*Specific PKC activity* (u mg^{-1} protein)			
		Not bound	*NaCl 1.0 M*	*NaCl 0.5 M*	*NaCl 0 M*	*Not bound*	*NaCl 1.0 M*	*NaCl 0.5 M*	*NaCl 0 M*
Rat brain	C	4±1	2±1	29±3	65±3	1.1±0.2	5.6±2.3	71.0±10.0	113.8±11.2
	M	0±0	1±0	16±3	83±3	0.1±0.1	0.8±0.2	23.3±6.7	111.4±35.2
Rat kidney	C	62±11	4±1	23±10	11±6	2.5±1.2	0.6±0.1	4.8±2.7	1.2±0.7
	M	50±13	4±1	21±5	25±8	1.5±0.3	0.9±0.2	4.2±1.3	3.7±1.2
Rat heart	C	21±5	16±1	38±3	25±5	0.3±0.1	3.2±0.5	7.1±1.0	3.3±0.8
	M	8±4	16±9	25±6	51±11	0.4±0.2	0.9±0.3	2.7±0.7	4.6±0.8
Rat aorta	C	2±2	24±4	15±13	59±3	0.3±0.3	1.3±0.3	0.7±0.0	3.3±0.4
	M	2±2	29±16	41±17	28±15	0.0±0.0	1.8±1.6	0.9±0.1	2.1±1.1
Human kidney	C	25±4	8±2	49±2	18±1	0.7±0.3	5.9±3.4	42.5±16.2	8.3±2.2
	M	7±2	3±0	23±7	67±9	1.0±0.2	1.3±0.2	12.0±5.2	35.4±12.2
Human heart	C	14±2	16±6	26±6	44±9	0.2±0.0	4.0±1.9	7.0±3.0	5.8±1.8
	M	14±9	3±2	18±5	68±7	2.6±2.0	0.3±0.2	0.7±0.3	4.9±1.9
Human aorta	C	11±3	7±2	24±3	59±5	0.0±0.0	0.6±0.3	1.3±0.5	2.6±0.4
	M	0±0	6±1	3±1	91±2	0.0±0.0	0.1±0.1	0.0±0.0	3.3±0.6
Human saphenous vein	C	16±3	20±1	17±3	49±5	0.2±0.1	5.3±1.0	4.5±1.3	5.8±0.3
	M	3±2	8±1	34±3	58±2	0.1±0.1	0.7±0.2	2.5±0.8	4.3±0.5

The DEAE purified (200 mM NaCl) cytosolic (C) and membrane (M) fractions were adjusted to 1.5 M NaCl and applied to phenylsepharose columns. The columns were washed with buffer containing 1.5 M NaCl and eluted stepwise with buffer containing NaCl 1.0 M, 0.5 M and 0 M. The left side of the table shows the relative PKC activity as % of total PKC activity recovered from the phenylsepharose columns taking into account the amount of protein eluted in the fractions and their specific activity (right side). The right side of the table shows the specific PKC activity in the different phenylsepharose fractions. Data are mean±s.e.mean of 4–5 independent experiments.

respectively). All other tissues had specific activities of less than 1 u mg^{-1} protein. Compared with the specific activity of the crude tissue extracts (data not shown) DEAE chromatography (200 mM NaCl fraction) enriched PKC activity 2–3 fold in cytosolic and 5–20 fold in membrane preparations.

In the next series of experiments we have investigated the purification of PKC activity upon phenylsepharose column chromatography. For this purpose the 200 mM NaCl fractions from DEAE chromatography were adjusted to 1.5 M NaCl and applied to phenylsepharose columns. The columns were washed with buffer containing 1.5 M NaCl and then eluted stepwise with buffer containing 1.0 M, 0.5 M and 0 M NaCl. The major fraction ($\geqslant$75%) of PKC activity from all tissues except for rat kidney (see below) bound to the phenylsepharose columns (Table 2). The bulk of PKC activity eluted with buffer containing 0.5 M NaCl or 0 M NaCl. Generally PKC activity in membrane extracts bound in a stronger manner to the hydrophobic phenylsepharose matrix than that from cytosolic extracts. The enrichment of PKC activity by phenylsepharose column chromatography varied considerably between tissues, e.g. 7 fold for rat brain cytosol and rat heart membrane extracts and 330 fold for human aorta membrane extracts. PKC from rat aorta was not enriched by phenylsepharose column chromatography (Table 2).

Using bisindolylmaleimide I, which inhibits all PKC isoforms, and Gö 6976, which specifically inhibits cPKC isoforms, we have evaluated which PKC class(es) contribute to functional PKC activity under our assay conditions. In experiments with combined cytosol and membrane fractions obtained from rat brain after phenylsepharose column chromatography both drugs completely inhibited PKC activity and had similar potencies (pIC_{50} 7.45 ± 0.18 and 7.66 ± 0.07, respectively; $n = 4$).

Additional experiments were designed to elucidate why a major fraction of rat renal PKC activity did not bind to phenylsepharose columns. Hence, we compared the activator profile for the rat kidney and brain PKC activity eluting with 1.5 M NaCl (not bound), 0.5 M NaCl and 0 M NaCl (Figure 1). The PKC activity in cytosol or membrane extracts of both

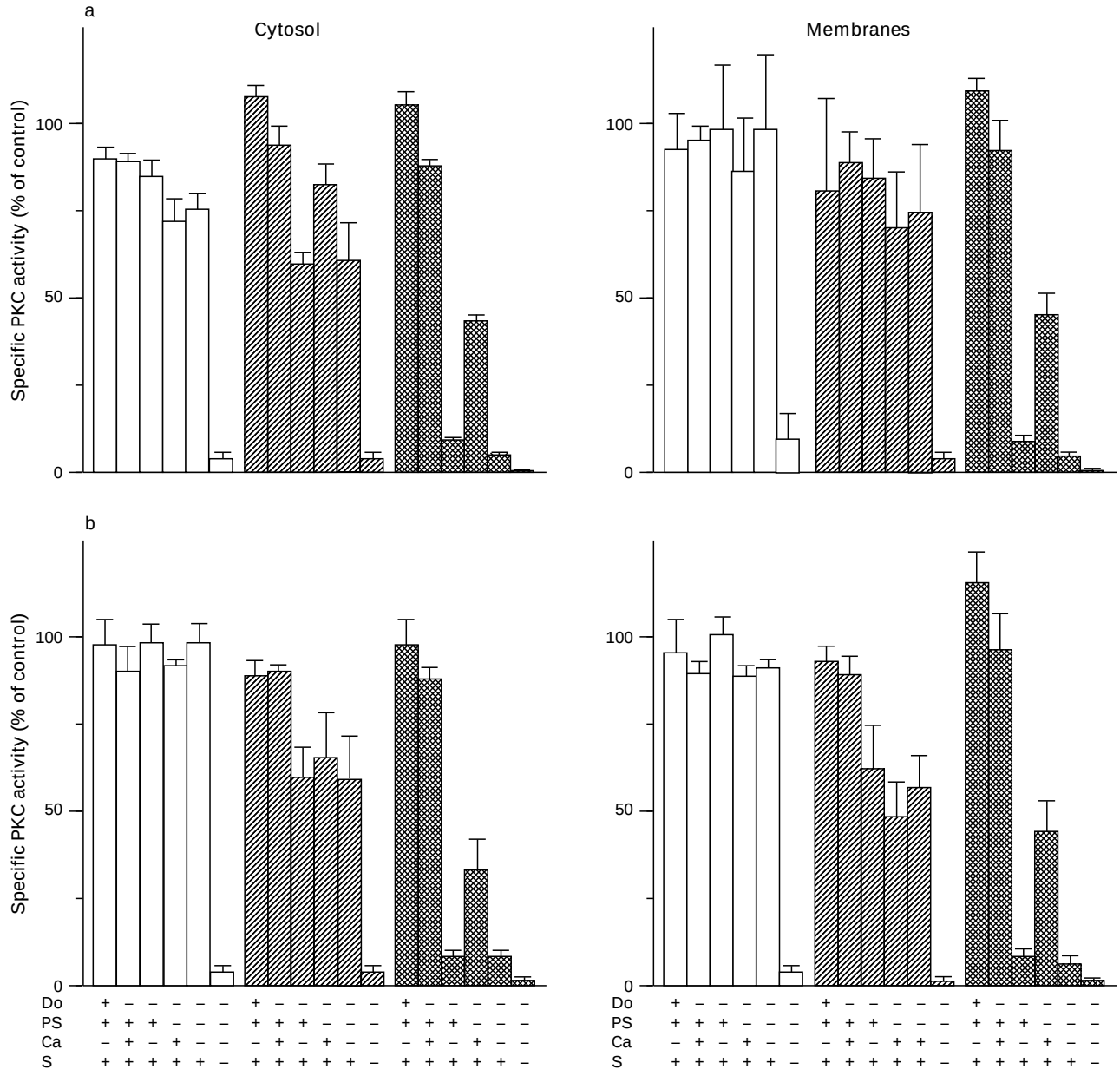

Figure 1 Purification of protein kinase C (PKC) activity from rat (a) brain and (b) kidney by phenylsepharose column chromatography. Specific PKC activity in different phenylsepharose fractions (open columns, not bound; hatched columns, NaCl 0.5 M; cross-hatched columns, NaCl 0 M) in the absence and presence of phosphatidylserine (PS), diolein (Do), Ca^{2+} (Ca) and the substrate [S^{25}]PKCα_{19-31} (S). The data were calculated as percentage of specific PKC activity in the presence of Do, PS, Ca and S (control). Data are mean $\pm$ s.e.mean of 3 independent experiments.

tissues, which did not bind to phenylsepharose, was not affected by removal of diolein, phosphatidylserine and/or Ca^{2+} from the assay. In contrast, the PKC activity in the 0 M NaCl fraction of both tissues strongly depended on the presence of phosphatidylserine and Ca^{2+} in the assay. The PKC in the 0.5 M NaCl fraction of cytosol and membranes in both tissues depended partially on the presence of the cPKC activators, phosphatidylserine and Ca^{2+}. Thus, the fraction of PKC, which did not bind to phenylsepharose, did not require phosphatidylserine for activation, indicating that it might represent a PKC activity which is not under the control of the regulatory subunit.

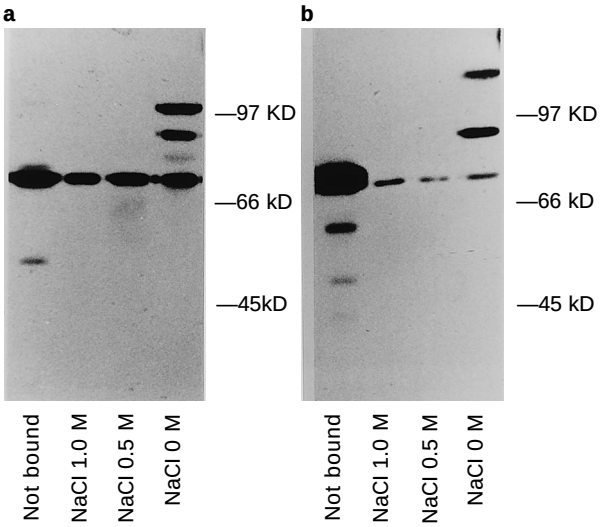

Figure 2 Purification of classical protein kinase C (cPKC) immunoreactivity from rat brain (a) and kidney (b) by phenylsepharose column chromatography. Western blot of different phenylsepharose fractions with an antibody against a common carboxyterminal region of cPKCs. Note that the isoform-specific antisera for the cPKC detection used in the other figures are not directed against the carboxy terminal but rather the V_3 regions.

If the PKC activity which does not bind to phenylsepharose lacks a regulatory subunit, it should have a smaller molecular weight. To investigate this possibility, we have compared PKC immunoreactivity in the four phenylsepharose fractions for cytosolic extracts of rat brain and kidney by Western blotting using an antibody which recognized the carboxy terminus of the three cPKCs α, β and γ (Figure 2). In the 0 M NaCl fraction from both tissues a major band with an apparent molecular weight of 80 kDa was detected, corresponding to the known size of cPKCs. An additional major band of more than 97 kDa was also detected in both tissues. Moreover, a band of 70 kDa was present in both tissues, and this band was also found in the other fractions. This band was most prominent in the fraction which did not bind to phenylsepharose in rat kidney. That fraction also contained a major band of 55 kDa in rat kidney but not in rat brain. These data are compatible with the idea that the phosphatidylserine-independent PKC activity represents a PKC which lacks a regulatory subunit.

Next, we investigated whether all PKC isoenzymes from rat brain and rat kidney behave similarly upon combined DEAE and phenylsepharose column chromatography. For this purpose, we determined immunoreactivity for ten PKC iso-

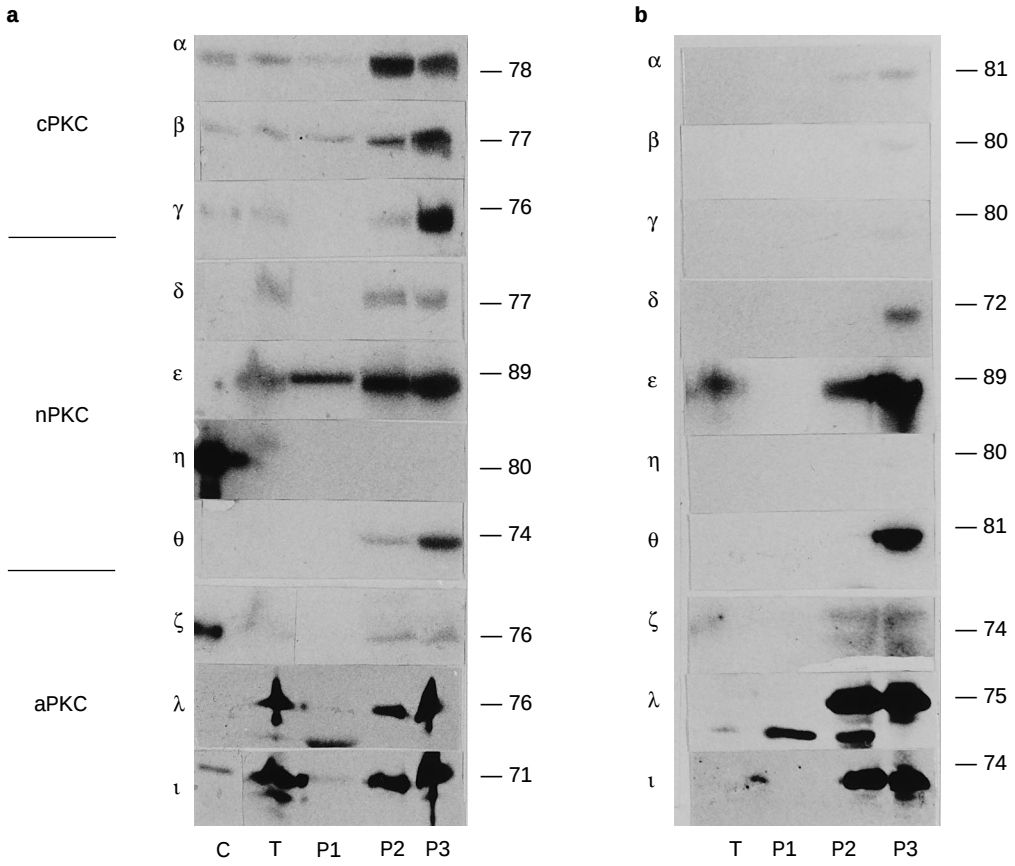

Figure 3 Purification of protein kinase C (PKC) immunoreactivity from rat brain (a, cytosolic and b, membrane fractions) by phenylsepharose column chromatography. PKC isoenzymes in raw protein extracts (T) and in phenylsepharose fractions eluting with 1.5 M NaCl (P1, not bound), 0.5 M NaCl (P2) and 0MNaCl (P3); 100 ng of recombinant human PKCα, β_{II}, γ, δ, η and ζ were used as controls (C). In the case of PKCλ and ι 10 μg of total rat brain or NIH3T3 cell lysates were used as controls (C), respectively. The recombinant human PKCδ control did not cross-react with the antibody raised against a sequence from rat PKCδ. The numbers on the right indicate the apparent molecular weight of the band of interest and were calculated from the molecular weight standards. Samples were pooled from 2 independent experiments.

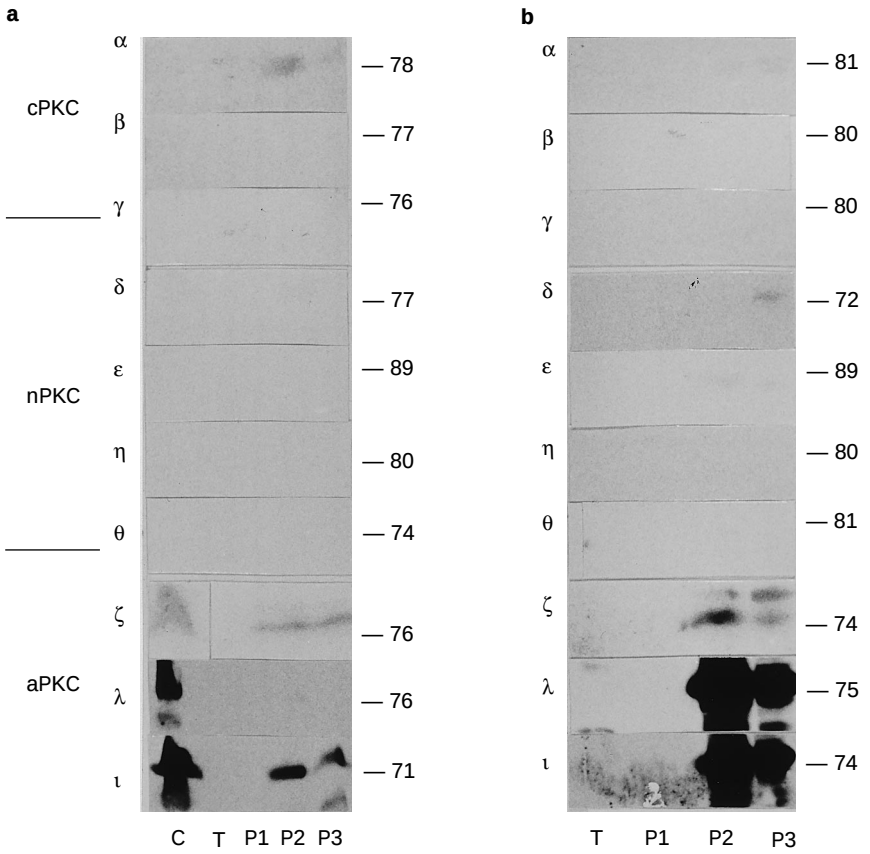

Figure 4 Purification of protein kinase C (PKC) immunoreactivity from rat kidney (a, cytosolic and b, membrane fractions) by phenylsepharose column chromatography. PKC isoenzymes in raw protein extracts (T) and in phenylsepharose fractions eluting with 1.5 M NaCl (P1, not bound), 0.5 M NaCl (P2) and 0MNaCl (P3). The numbers on the right indicate the apparent molecular weight of the band of interest and were calculated from the molecular weight standards. Samples were pooled from 2 independent experiments.

enzymes in crude tissue homogenates and in three phenylsepharose fractions (not bound, 0.5 M NaCl and 0 M NaCl) by use of isoenzyme specific antibodies (Figures 3 and 4). In rat brain PKCα, PKCβ, PKCγ, PKCδ, PKCε, PKCζ, PKCλ and PKCι were detected in cytosol extracts of crude tissue homogenates (Figure 3). PKCη was not detected in cytosolic or membrane extracts of crude tissue homogenates or in any of the phenylsepharose column elution fractions. All PKC isoenzymes, including PKCθ which had not been detectable in crude homogenates, were enriched in the fractions eluting from phenylsepharose with 0.5 M or 0 M NaCl. Only small amounts of cytosolic PKCα, PKCβ and PKCε did not bind to phenylsepharose (Figure 3). In membrane extracts from crude brain homogenates only PKCε was detected, indicating that membranes contained much less immunoreactive PKC than cytosol. However, various PKC isoforms were considerable enriched in the 0.5 M NaCl and 0 M NaCl fraction (Figure 3). All PKC isoforms found in cytosol were also found in membranes, but cPKCs were only faintly stained. In membranes PKCζ was detected as a double band.

In cytosolic extracts from crude rat kidney homogenates no PKC isoenzymes except the aPKCs, PKCζ, PKCλ and PKCι, were stained (Figure 4). Upon partial purification by combined column chromatography PKCα also became detectable in the 0.5 M and 0 M NaCl fraction. In these fractions PKCζ and PKCι were also detectable while PKCλ was not found. In membrane extracts from rat kidney raw tissue homogenates no PKC isoforms were stained by any of the antibodies used. Upon partial purification the aPKCs, PKCζ, PKCλ and PKCι, were considerably enriched and PKCα, PKCδ and PKCε became faintly detectable (Figure 4).

For all other tissues cytosolic and membrane extracts of crude tissue homogenates and of the 0 M NaCl fraction of the phenylsepharose columns were studied by immunoblotting. In rat heart cytosol no PKC isoenzymes except PKCλ and PKCι were detected (Figure 5). In rat heart membranes PKCδ, PKCε, PKCη, PKCθ and PKCζ were present in extracts from crude homogenates. Upon purification by phenylsepharose columns all signals increased, and PKCα, PKCλ and PKCι became additionally detectable (Figure 5).

In rat aorta cytosol no PKC isoenzymes except PKCλ and PKCι were detected (Figure 5). In membrane extracts from crude homogenates PKCδ, PKCζ, PKCλ and PKCι were found. After partial purification all enzymes were considerably enriched and PKCα, PKCε and PKCη were additionally stained (Figure 5). Like in rat brain and kidney membranes, PKCζ in rat heart and aorta membranes was detected as a double band although recombinant human PKCζ stained as a single band with an apparent molecular weight of 76 kDa.

In human kidney, PKCδ, PKCζ, PKCλ and PKCι were detectable in cytosolic extracts of crude homogenates but no major enrichment was found following phenylsepharose column chromatography (Figure 6). In membrane extracts of crude homogenates PKCα, PKCδ, PKCε, PKCη, PKCθ, PKCζ, PKCλ and PKCι were detectable, and all isoforms were enriched by phenylsepharose (Figure 6).

In human heart cytosolic extracts of crude homogenates or phenylsepharose eluates no PKC isoforms were detected (Figure 6). In membrane extracts of crude homogenates we also were unable to identify clearly any PKC isoform, but following partial purification by phenylsepharose PKCα, PKCδ, PKCε, and all three aPKC isoforms became detectable (Figure 6).

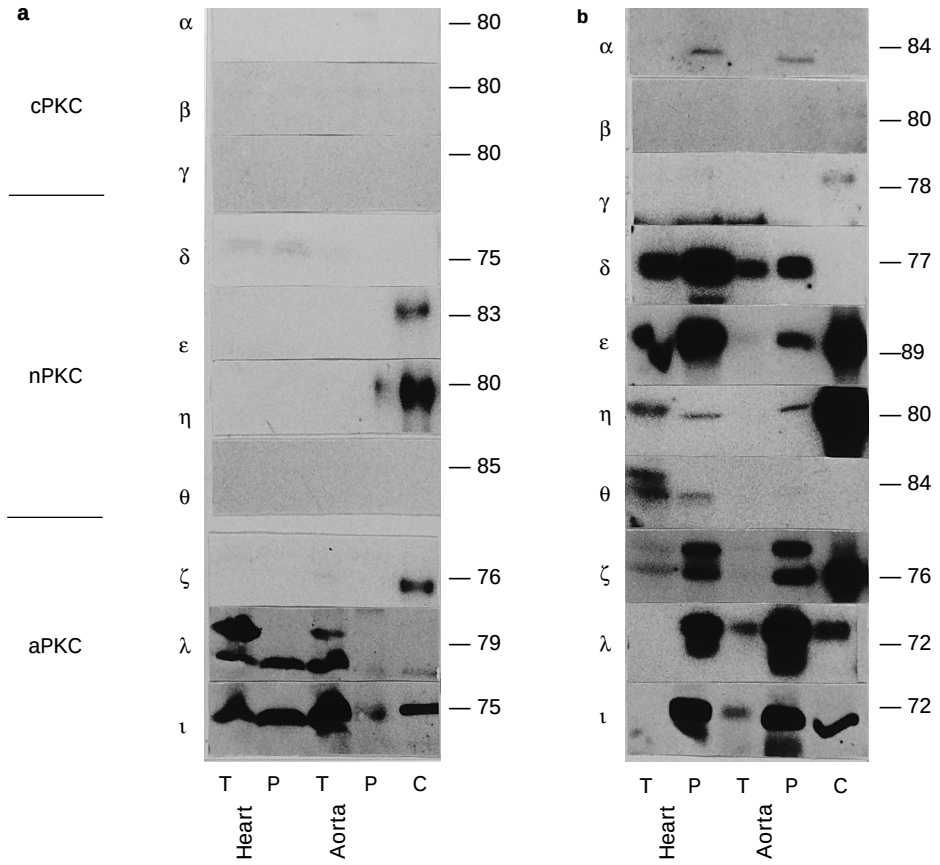

Figure 5 Protein kinase C (PKC) isoenzyme immunoreactivity in (a) cytosolic and (b) membrane fractions of rat heart and aorta. Raw protein extracts (T) or extracts purified by combined DEAE and phenylsepharose chromatography (P, O mM NaCl fraction) were analysed by PKC isoform specific antibodies; 100 ng of recombinant human PKCα, β_{II}, γ, δ, ϵ, η and ζ were used as controls (C). In the case of PKCλ and ι 10 μg of total rat brain or NIH3T3 cell lysates were used as controls (C), respectively. The recombinant human PKCδ control did not cross-react with the antibody raised against rat PKC. The numbers on the right indicate the apparent molecular weight of the band of interest in kDa and were calculated from the molecular weight standard curve. Samples were pooled from 2 independent experiments.

In human aorta cytosolic extracts of crude homogenates contained PKCλ and PKCι; they were not enriched upon phenylsepharose column chromatography, but PKCδ became additionally detectable. In membrane extracts of crude homogenates we also did not detect any PKC isoform, but PKCα, PKCδ, PKCε, and the three aPKC isoforms became detectable upon partial purification (Figure 6).

In human saphenous vein PKCδ, PKCλ and PKCι were detectable in cytosolic extracts of crude homogenates, and PKCδ but not the two aPKC isoforms were enriched by partial purification (Figure 6). In membrane extracts of crude homogenates no PKC isoform was detectable, but PKCα, PKCε and the three aPKC isoforms became detectable (Figure 6).

Discussion

We have determined the chromatographic behaviour of PKC activity and isoenzyme immunoreactivity from rat kidney, heart and aorta and human kidney, heart, aorta and saphenous vein upon combined DEAE and phenylsepharose column chromatography; rat brain, which contains all known PKC isoforms except PKCη (Osada *et al*., 1990; Bacher *et al*., 1992) was studied as a reference tissue. DEAE is an anion exchanger which enriches negatively charged molecules, while phenylsepharose enriches molecules with hydrophobic domains and thus is particularly suitable for the purification of membrane-bound proteins. Anion exchange chromatography with DEAE purified PKC activity from all tisues. The enrichment compared to crude homogenates was generally greater for membrane than for cytosolic extracts. While DEAE column chromatography was suitable for an initial purification of PKC activity from rat and human cardiovascular tissues, this partial purification occurred at the cost of a considerable recovery loss in some cytosolic but not membrane extracts of some tissues. In all tissues 70–90% of total tissue PKC activity eluted from the DEAE columns was found in the cytosolic extracts. Phenylsepharose columns bound 75–100% of DEAE-purified PKC from all tissues except rat kidney (see below), and thus provided further purification in most tissues. As with DEAE, phenylsepharose columns bound PKC from membranes in a somewhat stronger manner than cytosolic PKC.

PKC activity depended on the presence of Ca^{2+} and diolein, and the cPKC-specific inhibitor, Gö 6976 (Martiny-Baron *et al*., 1993), was similarly potent and effective as the general PKC inhibitor, bisindolylmaleimide. Thus, our functional assay may be biased towards cPKC isoforms. This preference may be related to a higher affinity of the substrate [Ser^{25}]PKCα_{19-31} for cPKC than aPKC or nPKC isoenzymes (Kazanietz *et al*., 1993). Therefore, the above conclusions may be limited to cPKCs.

Based on these limitations of the functional assay, we have studied whether immunoreactivity for all PKC isoforms behaves in a similar manner upon combined DEAE and phenylsepharose column chromatography. Rat brain cytosol and membranes contain all known PKC isoenzymes except PKCη (Osada *et al*., 1990; Bacher *et al*., 1992). Our immunoblots with isoform-specific antibodies demonstrate that indeed all PKC isoforms are considerably enriched by combined DEAE and phenylsepharose column chromatography in rat brain. However, the degree of enrichment may differ between isoforms,

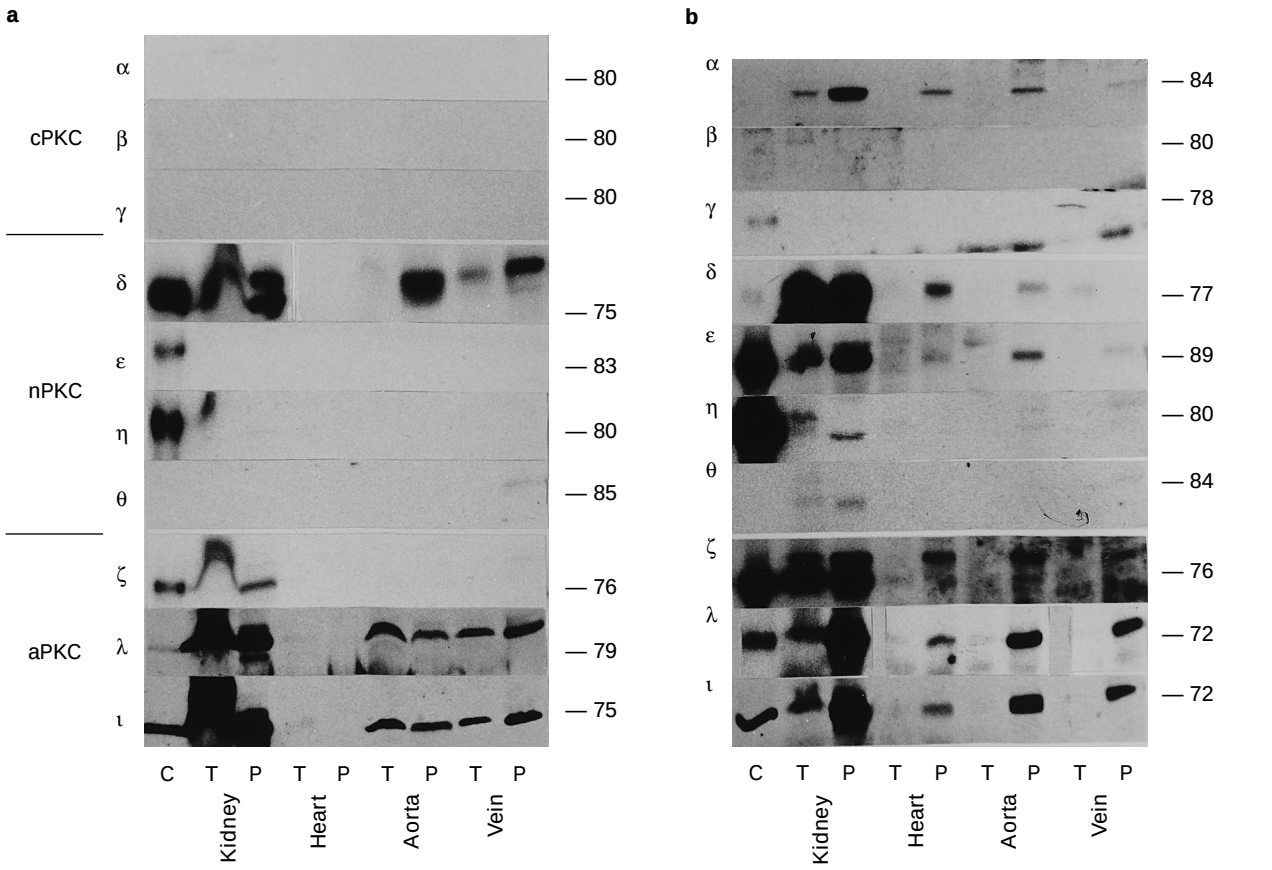

Figure 6 Protein kinase C (PKC) isoenzyme immunoreactivity in (a) cytosolic and (b) membrane fractions of human kidney, heart, aorta and saphenous vein (vein). Raw protein extracts (T) or extracts purified by combined DEAE and phenylsepharose chromatography (P, 0 mM NaCl fraction) were analysed by PKC isoform specific antibodies. 100 ng of recombinant human PKCα, β_{II}, γ, δ, ε, η and ζ were used as controls (C). In the case of PKCλ and ι 10 μg of total rat brain or NIH3T3 cell lysates were used as controls (C), respectively. In these experiments an anti PKCδ antibody with specificity for human PKCδ was used. The numbers on the right indicate the apparent molecular weight of the band of interest in kDa and were calculated from the molecular weight standard curve. Samples were pooled from 2 independent experiments.

particularly in cytosolic extracts, since aPKCsλ and ι were enriched much less than other PKCs. PKCη was detected in membranes from rat heart and aorta and was also found to be enriched upon partial purification. Thus, phenylsepharose columns are suitable for PKC isoenzyme purification, but this procedure may somewhat alter the detected ratios of isoenzymes.

In many tissues stronger immunoreactivity for PKC isoforms was detected in membrane extracts of crude homogenates than in cytosolic extracts. This apparent discrepancy with our functional data may, at least in part, be due to technical reasons. Thus, the antibodies used for detection of cPKC isoforms recognized the appropriate standard peptides less than the antibodies used for nPKC and aPKC detection, possibly indicating a lower affinity. Thus, the results from our activity assays may be biased towards cPKCs while those from our immunoblots may be biased towards aPKCs and nPKCs. Atypical PKC and nPKC isoforms may be less restricted to the cytosolic fraction under resting conditions than the cPKC isoforms.

PKC activity from rat kidney differed from that of all other tissues since its major fraction did not bind to phenylsepharose. Our data demonstrate that the PKC activity of rat kidney (similar to rat brain) eluting from phenylsepharose columns with 0 M NaCl is likely to be solely cPKC. In contrast PKC activity in the fraction which did not bind to phenyl-sepharose had an activator profile which was characteristic of a PKC fragment which is no longer under the control of the regulatory subunit, i.e. PKM (Cressman *et al*., 1995). These data indicate the possibility that the PKC activity not binding to phenylsepharose represents proteolytic fragments. A constitutively active PKCα form has also been detected by hydoxyapatite chromatography of PKC from rat brain (Allen *et al*., 1994). Accordingly the antibody against a common carboxyterminal region of all cPKCs detected bands with smaller molecular weights than to be expected for a cPKC in the fraction which did not bind to phenylsepharose, while one of the bands eluting with 0 M NaCl had the expected molecular weights of $\approx$80 kDa (Nishizuka, 1988). Since the low molecular weight bands were not seen in the phenylsepharose eluates when the isoform-specific antibodies directed against the V_3 regions were used, the proteolytic site is possibly located in the N-terminal part of the molecule which is consistent with the idea of PKM generation. Taken together, it appears that the lack of binding of PKC activity from rat kidney to phenylsepharose columns is due to proteolytic degradation of the enzyme, but antibodies directed against the V_3 region do not allow the identity of the isoform of PKC which is the substrate of the proteases to be determined. Based on our observations that (a) the activity assays predominantly recognize cPKC activity and (b) PKCα is the dominating isoform in rat kidney, we propose that the data obtained with cPKC-common antiserum mainly reflect enzymatic degradation of cPKCα. The resulting PKM binds less well to phenylsepharose than the holoenzymes. Therefore, phenylsepharose can be used to separate proteolytic PKC fragments.

Our experiments on PKC isoform expression patterns in various rat and human cardiovascular tissues allow two general conclusions: firstly, each tissue has a specific complement of PKC isoforms. This most likely reflects a cell type-specific

expression of the isoforms, and detection of a given isoform may largely depend on the abundance of certain cell types specifically expressing these isoforms. Secondly, rat and human tissue homologues in general appear to exhibit similar isoform expression patterns which confirms previous speculations (Nishizuka, 1988; Dekker & Parker, 1994). In the following we will discuss these conclusions for heart as a prototype tissue, since rat heart is probably the best investigated tissue in this respect.

The available data on PKC isoform expression in rat heart have recently been reviewed (Puceat & Brown, 1994; Clerk *et al*., 1995). Thus, PKCα has been found in whole heart, atria and ventricles. The present study confirms this observation for rat heart and extends it to human heart. While cardiac PKCα immunoreactivity at least partly comes from fibroblasts (Rybin & Steinberg, 1994), data showing whether it also exists in cardiomyocytes (Clerk *et al*., 1995) or not (Bogoyevitch *et al*., 1993; Rybin & Steinberg, 1994) are controversial. Results on the detection of cardiac PKCβ are inconsistent (Kosaka *et al*., 1988; Qu *et al*., 1991; Clerk *et al*., 1995), and the present study has also failed to find it in human or rat heart. When detected in whole heart, parallel detection in cardiac myocytes was not successful (Rybin & Steinberg, 1994) indicating a possible primary location of PKCβ in non-myocytes. As in the present study with rat and human heart, attempts to detect cardiac PKCγ expression have routinely failed (Clerk *et al*., 1995), and this isoform may be restricted to the brain. Taken together, PKCα may be the most abundant cPKC in heart but its existence in adult cardiomyocytes remains controversial. Thus, cPKC isoforms, which are believed to be activated upon neurotransmitter receptor coupling e.g. to phospholipase C, are only sparsely found in the terminally differentiated adult cardiomyocyte. Since PKCα is more readily detected in neonatal cardiomyocytes (Kohout & Rogers 1993; Rybin & Steinberg, 1994; Bogoyevitch *et al*., 1994; Disatnik *et al*., 1994; Puceat *et al*., 1994) we speculate that it could mainly be important in the developing myocardium and/or under pathophysiological conditions associated with hypertrophy development when a neonatal gene expression programme is reactivated.

Among the nPKCs PKCδ has routinely been found in rat heart at the protein and mRNA level (Church *et al*., 1993; Clerk *et al*., 1995) with few exceptions (Bogoyevitch *et al*., 1993). The present study confirms this and extends this observation to human heart, although we have only detected this isoform in the membrane fraction. While some investigators failed to detect PKCε in rat heart at the protein and mRNA level (Schaap *et al*., 1989; Wetsel *et al*., 1992; Church *et al*., 1993), it was found in most studies (Clerk *et al*. 1995). Two studies (Bogoyevitch *et al*., 1993; Rybin & Steinberg, 1994) even described PKCε to be the most abundant cardiac isoform. We have also detected it abundantly in rat heart but only in much smaller abundance in human heart. Similarly we detected PKCη in rat but not in human heart. PKCη m-RNA (Osada *et al*., 1990; Bacher *et al*., 1992) and immunoreactivity were also found in whole rat heart by others (Bogoyevitch *et al*., 1993) but do not appear to exist in isolated cardiac myocytes (Bogoyevitch *et al*., 1993). Taken together cardiac nPKC isoform expression may be somewhat species-dependent. Thus, rat heart mainly expresses PKCε while according to the present data in human heart mainly PKCδ is found, with PKCε being only faintly and PKCη not at all detectable.

Among the aPKC isoforms PKCζ has routinely been detected in cardiac tissue (Selbie *et al*., 1993; Akimoto *et al*., 1994; Clerk *et al*., 1995). The present study confirms this and also demonstrates it for human heart. The occurrence of a second higher molecular weight band with the anti PKCζ antiserum might be due to crossreactivity with cPKC isoforms (Allen *et al*., 1994). While one study has claimed that rat cardiac PKCζ is not found in adult cardiomyocytes (Rybin & Steinberg, 1994), other investigators have localized this isoform in cardiomyocytes of both neonatal (Kohout & Rogers, 1993; Bogoyevitch *et al*., 1994; Rybin & Steinberg, 1994; Puceat *et al*., 1994; Clerk *et al*., 1995) and adult heart (Bogoyevitch *et al*., 1993; Kohout & Rogers, 1993; Puceat *et al*., 1994). Much less is known about expression of PKCλ and PKCι, which may be species homologues, in cardiac tissue. Similarly mRNA for PKCλ and PKCι was described in mouse (Akimoto *et al*., 1994) and rat heart (Selbie *et al*., 1993). Due to their unique activation pattern the physiological role of the cardiac aPKC is presently unclear.

In summary, our data show that PKC activity in rat and human cardiovascular tissues can be considerably enriched upon combined DEAE and phenylsepharose column chromatography. In particular phenylsepharose chromatography can be used to eliminate constitutively active PKC, which may represent proteolytic fragments no longer under the control of the regulatory subunit and may be prominent, e.g. in rat kidney. All known PKC isoforms bind to DEAE and phenylsepharose columns and elute under similar conditons but quantitative differences may exist. Purification seems to be somewhat more efficient in membranes than in cytosol. While cPKC activity is mainly found in the cytosol, aPKC and nPKC may not be restricted to this cellular compartment. The pattern of PKC isoform expression is similar in rat and human tissues but distinct for each tissue and cell type. In rat and human cardiovascular tissues cPKCα, nPKCs, δ and ε, and aPKCs, ζ, λ and ι are ubiquiously expressed; in some tissues nPKCη and θ are additionally found, whereas the cPKCs β and γ are not detectable.

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