High Affinity Inositol 1,3,4,5-Tetrakisphosphate Receptor from Rat Liver Nuclei: Purification, Characterization, and Amino-Terminal Sequence†

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ABSTRACT: Inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) mediates nuclear calcium signaling [Köppler P., Matter, N., & Malviya A. N. (1993) *J. Biol. Chem. 268*, 26248−26252], and a distinct high affinity $InsP_4$ binding site is identified with rat liver nuclei [Köppler, P., Mersel, M., & Malviya, A. N. (1994) *Biochemistry 33*, 14707−14713] as compared with other rat liver membrane fractions. A novel $InsP_4$ receptor protein derived from rat liver nuclei has been purified to apparent homogeneity employing preparative isoelectric focusing, electrophoretic mobility, nondenaturating polyacrylamide gel electrophoresis, and electroelution. Isoelectric focusing indicated an isoelectric pH around 4.3 ± 0.2 which was further confirmed by bidimensional electrophoresis. The high affinity nuclear $InsP_4$ receptor was identified as a 74 kDa protein both on the SDS−PAGE and on the bidimensional electrophoresis. Partial microsequence analysis showed that the N-terminal end of nuclear $InsP_4$ receptor consists of amino acids: PNHKNEIAGNFS. The 74 kDa nuclear $InsP_4$ receptor protein is a distinct protein from the other $InsP_4$ receptors purified from other sources and documented in the literature.

Considerable interest has been generated in understanding second messenger function of inositol 1,3,4,5-tetrakisphosphate (Lückhoff & Clapham, 1992; Hill et al., 1988; Pittet et al., 1989; Yoo, 1991; Gure et al., 1992; Tsubokawa et al., 1994; Ely et al., 1990). In mammalian tissues inositol 1,3,4,5-tetrakisphosphate is formed by rapid phosphorylation of 1,4,5-$InsP_3$[1] by a specific 1,4,5-$InsP_3$ kinase (Irvine et al., 1986; Lee et al., 1990). Initially proposed (Irvine & Moor, 1987), concerted action of $InsP_4$ and $InsP_3$ on calcium influx has been supported (Wilcox et al., 1993; Fadool & Ache, 1994) as well as contested (Kuno & Gardner, 1987; Snyder et al., 1988). The action of $InsP_4$ in intracellular calcium mobilization (Morris et al., 1987; Hill et al., 1988; Yoo, 1991) and nuclear calcium signaling (Köppler et al., 1993) has been distinguished from that of the calcium mobilizing role of $InsP_3$ (Ely et al., 1990; Berridge, 1993; Malviya et al., 1990). High affinity and specific 1,3,4,5-$InsP_4$ binding sites, distinct from other inositol phosphates, have been found in a wide range of tissues (Irvine & Cullen, 1993). Subcellular distribution of selective 1,3,4,5-$InsP_4$ binding sites have been studied in rat liver membrane fractions (Köppler et al., 1994).

$InsP_4$ receptor proteins have been purified from pig cerebellum (Donié & Reiser, 1991), platelets (Cullen et al., 1995a), and rat cerebellum (Theibert et al., 1992). The $InsP_4$ receptors purified from pig cerebellum and platelet have a molecular masses of 42 and 104 kDa, respectively. From the rat cerebellum two distinct $InsP_4$-binding fractions were obtained (Theibert et al., 1992). One fraction contained a single binding protein of 182 kDa molecular mass, and the other fraction contained proteins of 174 and 84 kDa molecular mass possessing a high affinity for 1,3,4,5-$InsP_4$. The platelets $InsP_4$ has been cloned (Cullen et al., 1995b).

Our group has been engaged in understanding molecular basis for nuclear calcium signaling (Matter et al., 1993; Malviya & Block, 1993; Malviya, 1994), and here we report the purification to apparent homogeneity of a nuclear high affinity 1,3,4,5-$InsP_4$ receptor. The criteria exploited for purification of the nuclear $InsP_4$ receptor concerned its acidic isoelectric point. Thus the first step of purification consisted in preparative isoelectric focusing and refocusing in a liquid medium. $InsP_4$-binding protein was subsequently subjected to electrophoretic mobility at pH 8.8. Finally, receptor protein was electroeluted from a semipreparative polyacrylamide gel under nondenaturating conditions which on SDS−PAGE was revealed by silver staining as a 74 kDa protein. In this report, we document molecular characterization of the 74 kDa nuclear $InsP_4$ receptor and the 12 amino acid sequence present at the N-terminal end of nuclear $InsP_4$R.

MATERIALS AND METHODS

Materials. All the reagents were of highest purity and obtained from Sigma. Inositol phosphates were obtained from Boehringer (Germany). Inositol 1,3,4,6-P_4 was a kind gift from Dr. S-K. Chung (Korea). [^{3}H]IP_4 (specific activity 21 μCi/nmol) was purchased from DuPont, New England Nuclear (France).

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[1] Abbreviations: $InsP_4$, inositol 1,3,4,5-tetrakisphosphate; 1,3,4,6-$InsP_4$, inositol 1,3,4,6-tetrakisphosphate; 3,4,5,6-$InsP_4$, inositol 3,4,5,6-tetrakisphosphate; 1,3,4,5,6-$InsP_5$: inositol 1,3,4,5,6-pentakisphosphate; $InsP_6$, inositol 1,2,3,4,5,6-hexakisphosphate; 1,4,5-$InsP_3$, inositol 1,4,5-trisphosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*,*N*-tetracetic acid; $InsP_4$R, inositol 1,3,4,5-tetrakisphosphate receptor.

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Isolation of Rat Liver Nuclei and Solubilization of Nuclear Proteins. Rat liver nuclei were isolated as described earlier (Masmoudi et al., 1989). Briefly, small pieces of freshly removed rat liver were homogenized in 6−8 volumes of a medium containing 1.3 M sucrose, 1.0 mM $MgCl_2$, and 10 mM potassium phosphate, pH 6.8. After being filtered through four layers of cheesecloth, the homogenate was centrifuged for 15 min at 1000*g*. The pellet was suspended in a minimum volume of the homogenization medium and mixed with a medium containing 2.4 M sucrose so as to give a final 2.2 M sucrose concentration and centrifuged at 100 000*g* for 1 h. The final nuclear pellet was suspended in a medium containing 0.25 M sucrose, 5.0 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.5, and centrifuged for 10 min at 1000*g*. The resulting pellet was resuspended at a protein concentration of 10 mg/mL in a medium containing 50 mM Tris-HCl, pH 8.3, 0.3 M sucrose, 150 mM NaCl, 2 mM EDTA, 20% glycerol, 2% Triton X-100, 2 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. This was allowed to stand for 2 h on ice while stirring gently and centrifuged for 30 min at 12000*g*. The resulting supernatant served as the source for solubilized nuclear $InsP_4$ receptor which was the starting material for its purification.

Preparative Isoelectric Focusing of the Solubilized Nuclear Proteins. The solubilized nuclear proteins were subjected to isoelectric focusing using an isoelectric focusing preparative cell (Rotofor, Bio-Rad). The Rotofor was prefocused for 1 h, employing 40 mL of a medium containing 0.3 M sucrose, 0.2 mM EDTA, 20% glycerol, 2% Triton X-100 (Packard), and 2% (v/v) Biolytes, in a pH range from 3 to 10 (40% w/v BIO-RAD). Twenty-five milliliters of 0.1 M phosphoric acid was placed in the anionic chamber, and 25 mL of 0.1 M NaOH was placed in the cationic chamber. Solubilized nuclear proteins (4 mL) were added and focused during 2 h at 11 W constant power. At the end of electrofocusing, the voltage recorded was 1300−1400 V and the current was 7−8 mA. Subsequent to multiple preparative isoelectric focusings, the fractions showing highest $InsP_4$ specific binding activity were pooled and refocused (4 mL) for 2 h in 40 mL of a medium containing 0.3 M sucrose, and 0.2 mM EDTA, 2% Triton X-100, and 20% glycerol. At the end of this operation, voltage and current were recorded as 2000 V and 4 mA (power was 8 W), respectively.

Electrophoretic Mobility of the Nuclear $InsP_4$ Binding Protein. Since enriched nuclear $InsP_4$ receptor fractions displayed an isoelectric pH of 4.3 ± 0.2, electrophoresis of this material was carried out in an alkaline medium containing 50 mM Tris-HCl, pH 8.8, 20% glycerol, 1% Triton X-100, 150 mM NaCl, and 0.2 mM EDTA. Both anionic and cationic focusing chambers were filled with this buffer. A sample of 10 mL was injected at the cathodic end of the focusing chamber, and electrophoresis was carried out at 35 mA constant current for 15 min (150 V, 5 W). At the end of electrophoresis, fractions were harvested and tested for specific $InsP_4$ binding activity.

Micropreparative Electrophoresis in Nondenaturating Polyacrylamide Gel. The gels used for electrophoresis under nondenaturating conditions were of 1.5 mm thickness (6.5 × 8.5 cm) and moulded with a preparative comb (one reference well and one preparative well). Running gel (T% = 8.1, C% = 3.0) and stacking gel (T% = 8.1; C% = 3.0) were polymerized in 1.5 M Tris-HCl, pH 8.8, 1% ammonium persulfate (Bio-Rad), and 0.1% (v/v) TEMED (Sigma). Three hundred microliters of enriched $InsP_4$ receptor fraction obtained after gel filtration (detailed out under analytical electrophoresis, below) and containing 20% glycerol was loaded on the preparative well, and prestained BSA [10 μg in 20% glycerol, 0.05% (w/v); Coomassie blue and 0.01% (w/v) bromophenol blue] was loaded on the reference well. The electrophoresis buffer consisted of 25 mM Tris base and 192 mM glycine, pH 8.3 (electrophoresis buffer A). Electrophoresis was performed at 4 °C and turned off before elution of bromophenol blue (Mini Protean II, Bio-Rad).

Protein Electroelution from Semipreparative Gel. Subsequent to nondenaturating electrophoresis, a strip of the resolving gel was cut to detect proteins by silver staining and corresponding band of stained protein as well as control bands were sliced. The acrylamide gel strips were submitted to electroelution (electroeluter Model 422, Bio-Rad). Electroelution was done at 4 °C for 5 h (8 mA per gel strip) employing electrophoresis buffer A; the molecular weight cut-off of the dialysis membranes was 12−15 kDa. The pooled electroeluted material (2.5 mL) was dialyzed in a Slide-A-Lyser dialysis cassette (MERCK) against 5.0 mM bis-Tris HCl, pH 6.5, containing 0.2 mM EDTA for 5 h at 4 °C. The dialysate was concentrated by a factor of 10 in a speed VAC lyophilyser (Savant). In this way $InsP_4$ receptor protein was rendered into the actual binding medium.

Edman Degradation Microsequencing (N-Terminal Determination). The material electrophoresed by SDS−PAGE (see Analytical Electrophoresis below) was transferred to a PVDF (Amersham) strip using a semidry electrophoretic transfer cell (procedure described below) (Bio-Rad). The transfer buffer consisted of 25 mM Tris base, 192 mM glycine, 0.2% (w/v) SDS, and 10% methanol, and the transfer was carried out for 25 min at 20 V constant voltage (200−400 mA). The strip was stained for 2 min with a solution containing 0.25% (w/v) Coomassie Brilliant Blue R250, 10% acetic acid, and 40% methanol, and destained with several washings of the same solution devoid of Coomassie blue. The stained band was cut off and placed in a microsequencer (Biosystems Applied, Division of Perkin Elmer, Model 473 A).

Analytical Electrophoresis (a) SDS−PAGE. Prior to SDS−PAGE, the pooled protein fraction obtained after the electrophoretic mobility step was submitted (2.5 mL) to gel filtration chromatography using an Econo-Pac P6 cartridge (exclusion limit 6 kDa) attached to an Econo-system (Bio-Rad). The elution buffer contained 5.0 mM Tris-HCl, pH 8.3, and 0.2 mM EDTA, and the flow-rate was maintained at 1 mL/min. The eluted proteins were pooled in a volume of 4 mL and concentrated in a Speed-Vac lyophilizer by a factor of 10. A volume of 20 μL of this sample was mixed with 20 μL of sample buffer (Laemmli, 1970), boiled for 3 min, and loaded on a 1 mm thick SDS−PAGE gel (T% = 8.1, C% = 3.0 for resolving gel and T% 5.1; C% = 3.0 for stacking gel). The electrophoresis was carried out in a Mini-Protean II (Bio-Rad) electrophoresis system at 25 mA constant current. Proteins were silver stained according to Merril et al. (1984).

(b) Bidimensional Electrophoresis. Analytical isoelectric focusing was performed in capillaries containing polyacrylamide gel (T% = 7.0, C% = 3.0), 3.7% (v/v) Nonidet P40

(Sigma), and 2.5% (v/v) biolyte (in a pH range 3−10). The polymerized gel was overlayered with 10 μL of 8 M urea containing 5% (v/v) Nonidet P-40 and 1% biolyte, pH 3−10, (overlay buffer) and equilibrated with sample buffer [9.5 M urea, 2% (v/v) Nonidet P-40, 5% (v/v) β-mercaptoethanol, 0.4% (v/v) biolyte]. Isoelectric focusing was carried out at 500 V for 10 min, followed by 400 V overnight in a Mini-Protean II 2D (Bio-Rad). The gel was incubated in Laemmli sample buffer for 15 min and placed over SDS gel (T% = 7.0; C% = 3.0, 1 mm thickness).

[^{3}H]InsP$_4$ Binding Assay. [^{3}H]InsP$_4$ binding was carried out for 10 min at 0 °C (on ice) in a medium containing 50 mM bis-Tris-HCl, pH 6.5, and 2 mM EDTA. The final concentration of [^{3}H]InsP$_4$ was 2 nM, which gave about 10 000 cpm/assay (giving 25 000 cpm per pmol as specific activity). The binding was performed in an Eppendorf tube, and the final volume was 200 μL. Protein was precipitated (15 min on ice) by adding 300 μg of γ-globulin and 18% (final concentration) poly(ethylene glycol). The Eppendorf tubes were vortexed and centrifuged in a Beckman microfuge at 12 000 rpm at 4 °C for 5 min, and the supernatant was removed by aspiration. Any remaining fluid was removed with a Kleenex, and the pellet was solubilized with 500 μL of Soluene 350 (Packard) tissue solubilizer; 35 μL of glacial acetic acid was added, and the suspension was transferred to 4.0 mL of Rotiszint liquid scintillator (Roth) and radioactivity was determined by spectrometry counting (Beckman LS-6000 SC). Nonspecific binding was determined in the presence of 5 μM InsP$_4$.

Protein Determination. Protein was determined according to Bradford (1976), using Coomassie Plus Protein Assay Reagent (Pierce), employing BSA as a standard. When the protein concentration was not sufficient to give reliable results, proteins were determined by SDS−PAGE followed by silver staining, BSA serving as a standard.

RESULTS

Specificity of the Solubilized InsP$_4$ Binding Protein. Nuclear InsP$_4$-binding protein was solubilized with 2% Triton X-100 and 150 mM NaCl in an appropriate buffered medium. The solubilized protein at this stage displayed a 10-fold decreased ligand affinity for InsP$_4$ as compared with isolated nuclei. K_d for high affinity InsP$_4$ binding site was 18.6 ± 3 nM as compared with intact nucleus where K_d was 1.6 ± 0.2 nM (Köppler et al., 1993). Likewise, the low affinity binding site gave a K_d value of 640 ± 20 nM as compared with intact nucleus value 57 ± 6 nM. This may be attributed to the influence of solubilization medium (discussed further below). However, the ligand selectivity remained intact for InsP$_4$ over other inositol phosphates tested. IC$_{50}$ values are listed in Table 1 showing that 1,3,4,5-InsP$_4$ isomer was the most effective ligand.

Preparative Isoelectric Focusing of the Nuclear InsP$_4$ Binding Protein. The solubilized nuclear protein was submitted to isoelectric focusing (Figure 1). According to the profile obtained here, the isoelectric point of InsP$_4$ binding protein appeared between pH 4.5 and 5.0. A majority of the proteins were sorted out in a pH range from 6 to 9 (Figure 1 insert). During refocusing (Figure 2) InsP$_4$ binding protein appeared to move systematically toward acidic pH around 4.3. This indicated that the putative nuclear InsP$_4$ receptor protein was highly acidic. This particular characteristic of nuclear InsP$_4$ receptor proved advantageous in an effort to purify this receptor, since a majority of nuclear proteins were sorted out at neutral or alkaline pH. Furthermore, preparative isoelectric focusing in a liquid milieu provided high recovery as evaluated in terms of [^{3}H]InsP$_4$ binding activity, and there was a minimal loss of receptor proteins. When the acidic proteins (containing InsP$_4$-binding activity) were pooled and submitted to refocusing, no addition of new ampholytes was needed, since the latter were already present in the fractions harvested from the preceding step. Refocusing was achieved with a pH gradient from 3.4 to 6.4, which permitted higher resolution of the acidic nuclear proteins (Figure 2).

Electrophoretic Mobility of the Nuclear InsP$_4$ Receptor Protein. This step was carried out by electrophoresis (Figure 3) in an alkaline medium where acidic proteins became negatively charged and their respective mobility, under an electric field, was expected to be directly dependent on the density of charge that they carried. The InsP$_4$ binding protein isolated at this step was separated from other proteins due

Table 1: Pharmacology of InsP$_4$ Binding Protein in the Solubilized Nuclear Material[a]

inositol phosphate	IC$_{50}$ (nM)
1,3,4,5-InsP$_4$	19.6 ± 2.0
1,3,4,6-InsP$_4$	400 ± 43.0
3,4,5,6-InsP$_4$	90 ± 8.5
1,3,4,5,6-InsP$_5$	150 ± 16.2
InsP$_6$	210 ± 19.4
1,4,5-InsP$_3$	>1000

[a] The isolated rat liver nuclei were solubilized with 2% Triton X-100 plus 150 mM NaCl as described under Materials and Methods. The binding assay was done with 2 nM [^{3}H]InsP$_4$ and increasing concentrations of the competing ligand at 0 °C for 10 min. The details of binding assay are described under Materials and Methods. Results are the mean of three independent experiments.

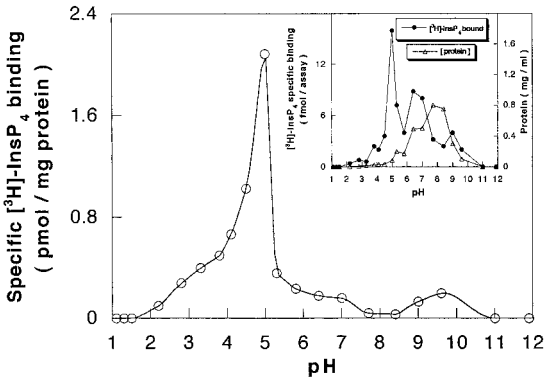

FIGURE 1: Isoelectric focusing of solubilized nuclear proteins. Nuclear proteins were subjected to isoelectric focusing for 2 h at 11 W constant power, in the Rotofor preparative cell as described under Materials and Methods. The Rotofor was prefocussed for 1 h. The anionic and cationic chamber-contained phosphoric acid and NaOH, respectively. At the end of isoelectric focusing the voltage recorded was 1300−1400 V and the current was 7−8 mA. Binding assays were performed at 0 °C for 10 min in Eppendorf tubes in a final volume of 200 μL, and [^{3}H]InsP$_4$ was 2 nM, which gave about 10 000 cpm per assay. Nonspecific binding was determined in the presence of 5 μM InsP$_4$. The figure shows a typical pH-dependent specific [^{3}H]InsP$_4$ binding per mg of protein. The insert depicts a profile of specific InsP$_4$ binding proteins derived from the isoelectric focusing step. The distribution of solubilized nuclear proteins separated at various pH is also shown in the insert. Fractions with InsP$_4$ binding activity of more than 10 000 cpm/mg of protein (4 < pH < 5) were pooled.

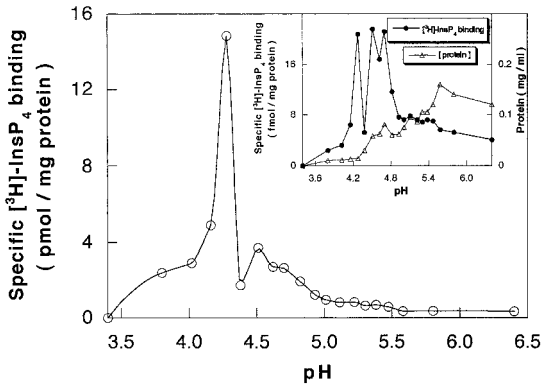

FIGURE 2: Refocusing of nuclear proteins. The pooled fractions as described to the legend to Figure 1 were refocussed in the Rotofor cell without additional ampholytes, thus permitting higher resolution of proteins in a narrow pH range. At this step two peaks of [^{3}H]-InsP_4 binding each with marked different affinity were separated on the basis of their apparent isoelectric pH. The proteins sorted out between isoelectric pH 4.4 and 4.8 were low affinity binding proteins (the nature of this protein is currently under investigation). The high affinity binding proteins were collected at pH 4.3 and were processed for further purification. The addition of 50 mM bis-Tris-HCl, pH 6.5, was sufficient to avoid any pH changes due to ampholytes.

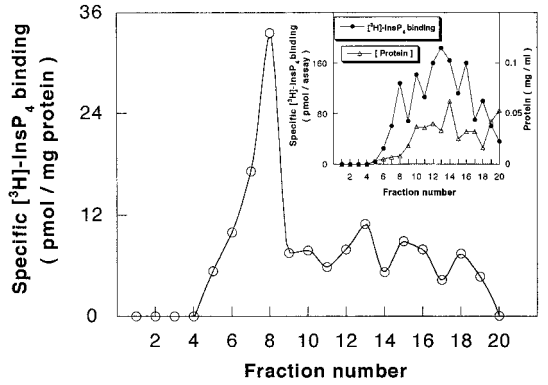

FIGURE 3: Electrophoretic mobility of nuclear InsP_4 binding proteins. Pooled fraction from isoelectric refocusing (Figure 2, pH 4.3 $\pm$ 0.2) were submitted to electrophoresis at 35 mA constant current for 15 min (150 V, 5 W) in an alkaline medium (pH 8.8), employing the Rotofor preparative cell and proteins were harvested for [^{3}H]InsP_4 binding assays, as described under Materials and Methods. Fractions were separated according to the density of charge carried by protein. Proteins collected towards the left hand side of the graph (anionic side of the Rotofor cell) were more negatively charged and hence moved faster. (Insert) fraction number 8 was highly enriched in InsP_4 binding and was a negatively charged protein.

to its fast electrophoretic mobility, but it was not totally free from contaminants as attested by SDS−PAGE analysis (Figure 5, lane D) as well as by the nondenaturating PAGE (Figure 4).

Micropreparative PAGE Studies. The enriched nuclear InsP_4 receptor was submitted to electrophoresis in a nondenaturating PAGE (without SDS), followed by electroelution. By this procedure macromolecules were separated as a function of their charge density and friction coefficient within the gel framework (Figure 4). Stacking gel as well as the running gel were prepared in an alkaline medium (pH, 8.8). This was necessary to obtain optimum condition permitting protein entry into the gel. Two protein bands (Figure 4A,B) were seen upon silver staining. Band A contained most of the InsP_4 binding activity. The band B was devoid of InsP_4 binding and appeared to be a contaminating protein. The

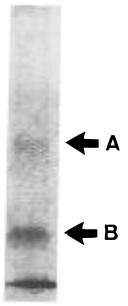

FIGURE 4: Electrophoresis under nondenaturating conditions. Proteins obtained after gel filtration chromatography (see analytical electrophoresis) were loaded on a semipreparative polyacrylamide gel (6.5 $\times$ 8.5 cm and 1.5 mm thickness) under nondenaturating conditions. The polyacrylamide gel was moulded with a preparative comb containing a reference well and a preparative well. This figure shows a part of the running gel which was cut for silver staining. Both protein bands (A and B) were electroeluted as described under Materials and Methods and InsP_4 binding was only associated with the band A. Protein loaded was 0.2 μg.

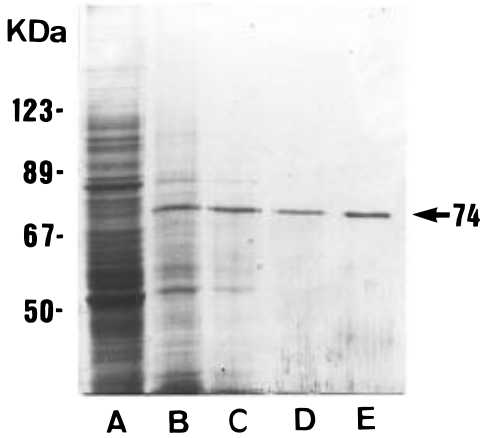

FIGURE 5: SDS−PAGE profile at various purification steps. Proteins obtained after each step of purification were loaded on a 1.5 mm thick SDS−polyacrylamide gel (T% = 8.1; C% = 3), as described under Materials and Methods section. Electrophoresis was carried out at 25 mA constant current, and proteins were silver stained. Each lane represents a step of purification: (lane A) Solubilized nuclear proteins (first step); (lane B) proteins pooled after first isoelectric focusing (second step); (lane C) proteins pooled after refocusing (third step); (lane D) proteins pooled after the electrophoretic mobility step (last but one step); and (lane E), protein obtained after electroelution from a semipreparative gel under nondenaturating conditions (final product, i.e., purified nuclear receptor). Numbers on the left-hand side indicates molecular mass in kDa (prestained SDS−PAGE marker from Sigma). On the right hand side arrow indicates 74 kDa nuclear InsP_4 receptor. The amount of protein loaded on each lane was 0.1−1.0 μg.

band A was electroeluted from nondenaturating gel and characterized as high affinity nuclear InsP_4 receptor having a molecular mass on SDS−PAGE as 74 kDa (Figure 5, lane E).

SDS−PAGE Analysis. Figure 5 illustrates SDS−PAGE analysis of materials derived at various steps of purification (Table 2). Lane A depicts solubilized nuclear proteins. The SDS−PAGE data are compatible with the isoelectric focusing results. For instance, during isoelectric focusing (Figure 1) a majority of proteins were sorted out which was further confirmed by SDS−PAGE (Figure 5, lanes B and C). The electrophoretic mobility step (Figure 5, lane D), although showing 74 kDa protein, apparently, without contaminations, upon nondenaturating PAGE analysis revealed that there was an additional protein (Figure 4B) associated with the receptor

Table 2: Summary of Purification Profile of High Affinity Nuclear 1,3,4,5-$InsP_4$ Receptor[a]

purification steps	total protein (mg)	specific binding (pmol/mg of protein)	total binding (pmol)	recovery (%)	fold of purification
solubilized nuclei	125	0.08	10.2	100	1
isoelectric focusing I	4.4	1.7	7.5	72.0	21
isoelectric focusing II	0.45	12.4	5.6	55.0	152
electrophoretic mobility	0.034	29.4	1.1	10.9	401
electroelution	0.0015	125.0	0.19	1.8	1522

[a] The solubilization of nuclear proteins were done with 2% Triton X-100 and 150 mM NaCl in an appropriate buffer. The solubilized material was subjected to isoelectric focusing in the pH range of 1−12. The same procedure was repeated for pH ranging from 3.4 to 6.4. In a second step the pooled fraction was subjected to free electrophoresis which separated proteins based on their charge density. Electroelution was finally carried out from micropreparative nondenaturating PAGE. Details of each step are described under Materials and Methods. These data are derived from a single experiment typical of at least three different purifications. The concentration of [^{3}H]$InsP_4$ was 2 nM, and nonspecific binding was determined in the presence of 5 μM $InsP_4$. The 100% of the total binding represents the actual binding on the starting material.

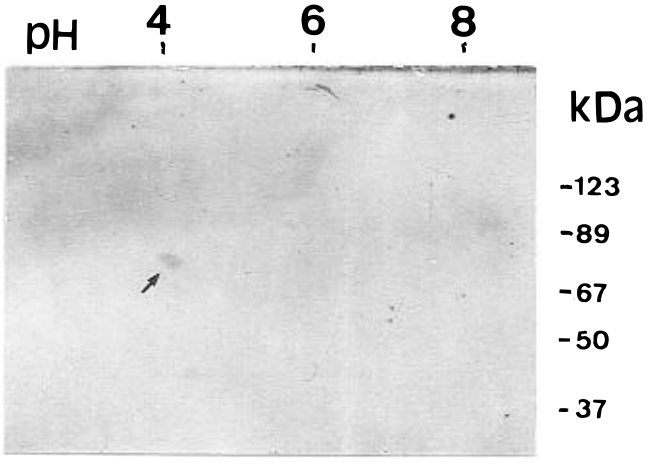

FIGURE 6: Bidimensional electrophoresis was carried out with 50 ng of purified receptor as described under Materials and Methods. The arrow indicates silver stained nuclear $InsP_4$ receptor, with a molecular mass of 74 kDa and isoelectric pH around 4.3.

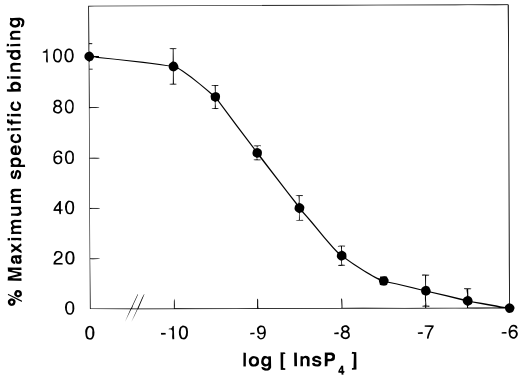

FIGURE 7: 74 kDa receptor [^{3}H]$InsP_4$ binding. The $InsP_4$ receptor purified from rat liver nuclei was assayed for [^{3}H]$InsP_4$ binding for 10 min at 0 °C. Binding assays were performed in Eppendorf tubes in a final volume of 200 μL. [^{3}H]$InsP_4$ was 2 nM and gave about 10 000 cpm/assay (specific activity 25 000 cpm per pmol). Nonspecific binding was determined in the presence of 5 μM $InsP_4$. Bound and free radioligands were separated by centrifugation.

protein. The $InsP_4$ receptor is shown in Figure 5, lane E, as a 74 kDa protein band, purified to apparent homogeneity.

Bidimensional Electrophoresis Analysis. Analytical 2D electrophoresis (Figure 6) of the purified $InsP_4$ receptor protein confirmed the apparent molecular mass of 74 kDa and the isoelectric pH in the vicinity of 4.3.

Characteristics of Nuclear $InsP_4$ Receptor. Fractions obtained after gel filtration chromatography were submitted to nondenaturating polyacrylamide gel electrophoresis (without SDS), followed by electroelution. The material recovered after electroelution was dialyzed against 10-fold diluted binding buffer. This step was necessary to render the purified receptor into the actual binding medium. High affinity binding properties of the receptor were (Figure 7) supported by the displacement of [^{3}H]$InsP_4$ by 1,3,4,5 $InsP_4$. The SDS−PAGE showed that nuclear $InsP_4$ receptor has a molecular mass of 74 kDa (Figure 5, lane E). The isoelectric pH of this receptor was 4.3 ± 0.2. The N-terminal microsequencing of $InsP_4$ receptor gave following amino acid sequence: Pro-Asn-His-Lys-Asn-Glu-Ile-Ala-Gly-Asn-Phe-Ser.

DISCUSSION

Subcellular distribution of 1,3,4,5-$InsP_4$ binding in rat liver membrane fractions (Köppler et al., 1994) revealed that the nuclei were endowed with high affinity and low affinity 1,3,4,5-$InsP_4$ binding sites (Köppler et al., 1993). In the present paper, we achieved purification of nuclear high affinity $InsP_4$ binding protein. The solubilized nuclear material displayed characteristics for both high and low affinity binding sites and ligand specificity for 1,3,4,5-$InsP_4$, except that K_d for both sites were higher as compared with isolated nuclei (Köppler et al., 1993). A high K_d value upon solubilization of receptor may be attributed to the medium containing detergent and salt employed for solubilization which was the first step at which receptor was rendered into an environment different from its native state. This caused decreased ligand affinity to the receptor and hence high K_d values at this stage. Similar decreased ligand−receptor affinity was seen (Humbert et al., 1996) when nuclei were treated with sodium citrate used to separate membranes of nuclear envelope.

The solubilized nuclear material was analyzed in terms of IC_{50} with various inositol phosphates (Table 1). The rank order of [^{3}H]$InsP_4$ binding inhibition was 1,3,4,5-$InsP_4$, 3,4,5,6-$InsP_4$, 1,3,4,5,6-$InsP_5$, $InsP_6$, 1,3,4,6-$InsP_4$, and 1,4,5-$InsP_3$. This confirmed that the nuclear $InsP_4$ binding protein dealt here was selective and specific to 1,3,4,5-$InsP_4$ isomer. We have taken approach in this study to separate receptor protein based on its isoelectric pH and charge density by using the same apparatus (i.e., Rotofor). The first purification step (Figure 1) consisted of preparative isoelectric focusing. Fractions, separated at acidic pH, contained proteins highly enriched in $InsP_4$ binding activity indicating a highly acidic nature of putative $InsP_4$ receptor. Fractions were pooled and refocused (Figure 2) with narrowed pH gradient (pH 3.4−6.4). This resulted in resolving further acidic proteins endowed with selective $InsP_4$ binding char-

acteristics. This step also facilitated to pool the high affinity binding protein which was separated from the low affinity $InsP_4$ binding material (Figure 2 insert). The low-affinity binding proteins possessed a K_d of 67 ± 10 nM and an isoelectric point of 4.5−4.8 (data not shown) and were eliminated at this step.

The subsequent step of purification (Figure 3) concerned with the electrophoretic mobility again using Rotofor cell. At this step proteins were separated on the basis of their molecular mass and their charge density. The ultimate step of purification was achieved by PAGE under nondenaturating conditions followed by electroelution of protein. Analytical 2D electrophoresis confirmed the isoelectric pH 4.3 ± 0.2 and a molecular mass of 74 kDa of the purified $InsP_4$ receptor protein (Figure 6). The partial amino-terminal sequence was carried out using electroeluted 74 kDa $InsP_4$ receptor. Microsequencing gave following N-terminal amino acid sequence (single letter code): PNHKNEIAGNFS.

A summary of purification steps is presented in Table 2. The success of purification at each step is fully supported by the data of $InsP_4$ binding specific activity. The enriched nuclear $InsP_4$ receptor protein exhibited similar characteristics in displacing [^{3}H]$InsP_4$ by cold $InsP_4$ (Figure 7) as was observed with isolated nuclear preparations (Köppler et al., 1994). The monophasic Scatchard analysis (Humbert et al., 1996) of saturation experiments observed with purified outer membrane and inner membrane indicated that the $InsP_4$ affinity characteristics may be consequent to their discrete distribution to the two membranes.

The data documented here regarding the nuclear $InsP_4$ receptor protein are the first of their kind, and it is not possible to evaluate at this stage how the nuclear receptor purified here relates with the other $InsP_4$ receptor purified earlier with a similar pharmacology from cerebellum (Donié & Reiser, 1991; Theibert et al., 1992) or platelets (Cullen et al., 1995a). From the pig cerebellum (Donié & Reiser, 1991) a protein of 42 kDa has been purified as $InsP_4$ receptor, whereas from the rat cerebellum (Theibert et al., 1992) proteins of 84, 174, and 182 kDa have been purified and shown to be putative $InsP_4$ receptor. Platelets, which are anucleated cells, have 104 kDa protein as $InsP_4$ receptor (Cullen et al., 1995a). In what manner proteins of multiple molecular mass from a variety of sources (neuronal vs non neuronal) are related with each other shall have to be evaluated by molecular cloning studies of these proteins. The sequence and molecular cloning of 104 kDa platelets $InsP_4R$ has been published (Cullen et al., 1995b). This protein appears to be a member of the family of GTPase- activating proteins, and hence a relationship between platelets $InsP_4R$ and activation of Ras is proposed (Cullen et al., 1995b). The N-terminal amino acid sequence of the platelets $InsP_4R$ is quite different from the N-terminal sequence of nuclear receptor that we document in this paper. At the level of plasma membranes the intervention of $InsP_4$, involving G proteins in calcium entry is understandable. But in what manner G proteins intervene with nuclear calcium signaling is difficult to apprehend in view of prevailing lack of knowledge concerning the location and function of Ras related proteins in the nucleus. Out of various Ras related proteins (Block & Wittinghofer, 1995), Ran is located to the nucleus (Ren et al., 1993) and is implicated in protein export from the nucleus (Moroinau & Blobel, 1995). However, the role of Ran in nuclear calcium signaling seems less likely since Ran remains unimplicated (Sazer, 1996) in cellular calcium signals.

The purification and characterization of nuclear $InsP_4$ receptor fully vindicates the proposed action of $InsP_4$ in nuclear calcium signaling (Köppler et al., 1993). Considering cellular function of $InsP_4$, two other important roles have been proposed, (i) regulation of calcium entry through the plasma membrane (Morris et al., 1987), and (ii) resequestration of calcium into internal stores (Hill et al., 1988; Yoo, 1991). Obviously, $InsP_4$ seems to mediate cellular calcium signaling at multiple locations in a cell and each one of cellular compartment appears to be endowed with specific and selective $InsP_4$ receptor. There are indications that certain calcium channels may be activated by $InsP_4$ (Fadool & Ache, 1994). Whether $InsP_4R$ serves as a calcium gated channel shall be required to be addressed by researches in the future. Purification of receptor protein from various organelles will provide useful tools to address the role of $InsP_4$ in calcium mobilization from parasitic protozoan (Raha et al., 1995) to human (Parent & Quirion, 1994).

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