Subcellular Localization of Specific Inositol 1,3,4,5-Tetrakis([^{3}H]phosphate) Binding Sites in Rat Liver Membrane Fractions: A Comparative Evaluation of pH Sensitivity and Binding Characteristics†

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ABSTRACT: Inositol 1,3,4,5-tetrakis([^{3}H]phosphate) ([^{3}H]IP_4) binding sites were investigated in plasma membranes, nuclei and microsomes derived from the rat liver. The pH optimum for maximum [^{3}H]IP_4 binding was not the same for plasma membranes, pH 7.5, nuclei, pH 6.5, and microsomes, pH 8.0. Evidence is presented demonstrating that inositol 1,3,4,5-tetrakis(phosphate) (IP_4) was the most effective inositol phosphate in displacing the binding of the [^{3}H]IP_4 in all the membrane fractions studied. Furthermore, the rank order of inhibition in various membrane fractions was identical; i.e., IP_5, Ins(3,4,5,6), and IP_3. This suggests that similar types of putative IP_4 receptor proteins are dealt with in the plasma membranes, nuclei, and microsomes. Scatchard analysis of saturation isotherms revealed a single binding site in the plasma membranes and in the microsomes, whereas two binding sites marked by distinct K_D and B_{max} values were found in the nuclei. The density of putative IP_4 binding sites in the plasma membranes corresponded to that of the high-affinity ones in the nuclei. Microsomes contained fewer binding sites as compared with plasma membranes or nuclei. On the basis of the pH sensitivity of [^{3}H]IP_4 binding and the K_D and B_{max} values in various membrane compartments, it is proposed that inositol 1,3,4,5-tetrakis(phosphate) receptor proteins are similar but not identical in membrane fractions in rat liver. Plasma membrane [^{3}H]IP_4 binding was displaced with IP_4 and IP_6, revealing IC_{50} values of 8 ± 2 and 150 ± 20 nM, respectively, indicating that rat liver plasma membrane IP_4 receptor is not clathrin assembly protein AP-2. This study provides a pH-dependent regulatory mechanism for the mode of functioning of IP_4 in different membrane fractions, which may be relevant for pH and calcium interactions during cellular growth promotion.

Considerable progress has been made in understanding the mechanism by which phosphatidylinositol produces second messenger molecules. It is very well established (Berridge, 1993) that one of the key events in intracellular signaling is the receptor-mediated hydrolysis of phosphatidylinositol 4,5-bis(phosphate) by phosphoinositidase C, generating inositol 1,4,5-tris(phosphate) (IP_3),[1] which binds to its receptor (IP_3R) at a specific cellular location and thus evokes intracellular calcium mobilization. IP_3 is also converted to inositol 1,3,4,5-tetrakis(phosphate) (IP_4) by the action of IP_3-3-kinase (Connolly et al., 1987; Choi et al., 1990). Bradford and Irvine (1987) were the first to demonstrate in HL60 cell membranes saturable inositol 1,3,4,5-tetrakis(phosphate) specific binding sites that were selective for IP_4 over other inositol phosphate derivatives. Subsequently, inositol 1,3,4,5-tetrakis(phosphate) binding sites have been reported in a variety of neuronal and nonneuronal tissues (Enyedi & Williams, 1988; Enyedi et al., 1989; Challiss et al., 1991; Donié & Reiser, 1989; Cullen & Irvine, 1992). A remarkably sharp difference in optimum pH requirement for inositol 1,3,4,5-tetrakis(phosphate) binding has been observed (Challiss et al., 1991; Donié & Reiser, 1989; Theibert et al., 1987), and proteins of varying molecular mass have been shown to bind IP_4 (Theibert et al., 1991; Donié & Reiser, 1991). Inositol 1,3,4,5-tetrakis(phosphate) receptor (IP_4R) has been purified from rat (Theibert et al., 1991) and pig (Donié & Reiser, 1991) cerebellum. Considerable work has been carried out over the last few years to characterize the nature of putative IP_4 binding receptor proteins, but no such study of the rat liver has been reported. We (Köppler et al., 1993) have demonstrated saturable inositol 1,3,4,5-tetrakis(phosphate) specific binding sites at pH optimum 6.5 on purified rat liver nuclei and documented that IP_4 mediates calcium uptake in the isolated nuclei. This study prompted us to characterize in detail the subcellular distribution of specific inositol 1,3,4,5-tetrakis([^{3}H]phosphate) binding sites over other inositol phosphates in rat liver. Insight into the function of inositol phosphates can be obtained by characterizing their binding proteins in different cell compartments. We report in this paper that there is an organelle-specific pH dependence of IP_4 binding sites in rat liver membrane fractions, which provides a reason to suggest that the nature of IP_4

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[1] Abbreviations: IP_3, inositol 1,4,5-tris(phosphate); IP_4, inositol 1,3,4,5-tetrakis(phosphate); IP_5, inositol 1,3,4,5,6-pentakis(phosphate); Ins(3,4,5,6), inositol 3,4,5,6-tetrakis(phosphate); IP_6, inositol 1,2,3,4,5,6-hexakis(phosphate); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; IP_3R, inositol 1,4,5-tris(phosphate) receptor; IP_4R, inositol 1,3,4,5-tetrakis(phosphate) receptor; MES, 2-(*N*-morpholino)ethanesulfonic acid.

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binding protein in various subcellular locations in the rat liver may be similar but not identical. Data are presented demonstrating that the plasma membrane and nuclear IP_4 receptor (at least the high-affinity one) proteins are not inherently different, which attests to the role of IP_4 receptor in gating calcium fluxes (Malviya, 1994; Willcox et al., 1993) at these locations. Furthermore, a comparison of the IC_{50} values of IP_4 and IP_6 revealed that the rat liver plasma membrane IP_4 receptor protein is not clathrin assembly protein AP-2 (Voglmaier et al., 1992).

MATERIALS AND METHODS

Materials. [^{3}H]IP_4 was purchased from DuPont/New England Nuclear (France). IP_3, IP_4, Ins(3,4,5,6), and IP_5 were obtained from Boehringer (Germany). All other chemicals, including IP_6, were from Sigma, France, and were of highest purity.

Preparation of Rat Liver Subcellular Fractions. Mitochondria, microsomes, and plasma membranes were prepared as described earlier (Malviya et al., 1990) except that plasma membranes were further purified by employing sucrose gradient centrifugation. Briefly, small pieces of rat liver were homogenized in 8 vol of a medium containing 0.3 M sucrose, 1.0 mM EDTA, and 10 mM Tris-HCl, pH 7.5 (medium A). The homogenate was filtered through four layers of cheesecloth and centrifuged for 5 min at 746*g*. The pellet, which contained crude nuclei and cell debris, was washed by suspending it in medium A and centrifuging as before. This procedure was repeated twice. Washings were mixed with the supernatant from the previous step. The sedimented material was washed a third time, and the resulting supernatant was mixed with the post-plasma-membrane fraction (see below). The mixed postnuclear supernatant was centrifuged for 10 min at 8000*g*. The pelleted mitochondria were washed in an appropriate volume of medium A by centrifuging for 15 min at 17000*g*. Postmitochondrial supernatant plus subsequent washes were centrifuged for 20 min at 35000*g*. The resulting pellet was the plasma membrane fraction, and it was suspended in medium A containing 1.45 M sucrose. This suspension was placed in a centrifuge tube and overlayered with medium A. This was centrifuged for 1 h at 100000*g*; the plasma membrane was recovered at the interface of the two media. The purified plasma membrane was suspended in medium A and centrifuged for 15 min at 35000*g*. The supernatant obtained from the previous 35000*g* centrifugation, the supernatant from the third washing of the first 746*g* pellet, and the post-8000*g* supernatant were mixed and centrifuged for 1 h at 180000*g*. The resulting pellet gave the microsomal fraction, which was suspended in an appropriate volume of medium A and recentrifuged for 1 h at 180000*g*. The final pellet served as the microsomal fraction, which was suspended in an appropriate medium. The supernatant of the first 180000*g* fraction served as the cytosolic fraction. The entire operation was carried out at 4 °C.

Rat liver nuclei were prepared as described by Masmoudi et al. (1989). Briefly, small pieces of liver freshly removed from rat were homogenized in 8 vol of a medium containing 1.3 M sucrose, 1.0 mM $MgCl_2$, and 10 mM potassium phosphate, pH 6.8. The homogenate was filtered through four layers of cheesecloth and centrifuged for 15 min at 1000*g*. The resulting pellet was suspended in an appropriate volume of homogenization medium. This suspension was mixed with a medium containing 2.4 M sucrose, 1 mM $MgCl_2$, and 10 mM potassium phosphate, pH 6.8, to give a final 2.2 M sucrose concentration, which was checked with a refractometer, and the mixture was centrifuged for 1 h at 100000*g*. The resulting 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 pellet was the final nuclear preparation free of any microsomal (Malviya et al., 1990) or plasma membrane (Masmoudi et al., 1989) constituents as attested by the marker enzyme activity. Protein was determined according to Bradford (1976) with bovine serum albumin employed as a standard.

Inositol 1,3,4,5-Tetrakis([^{3}H]phosphate) Binding. For the study of [^{3}H]IP_4 binding at pH 6.0 or 6.5, 10 mM MES buffer was used. For the neutral and alkaline pH binding, 50 mM Tris-HCl was used. The binding assay medium contained 100 mM KCl, 20 mM NaCl, and 1 mM EDTA. The binding assay was performed at a protein concentration of 0.4−1.0 mg per assay at 0 °C (on ice) for 20 min in a final volume of 400 μL in an Eppendorf centrifuge tube. The concentration of [^{3}H]IP_4 (specific activity, 21 μCi/nmol) was 1 or 2 nM. Nonspecific binding was determined in the presence of 10 μM IP_4. In the case of homogenate, nuclei, plasma membranes, and mitochondria the bound and free [^{3}H]IP_4 were separated by centrifugation (Beckman Microfuge) for 15 min at 12 000 rpm, and the supernatant was removed by aspiration. Any remaining fluid was removed with a Kleenex, and the pellet was solubilized in 1 mL of Soluene 350 (Packard) tissue solubilizer. Seventy microliters of glacial acetic acid was added, the suspension was transferred to 5-mL Biofluor liquid scintillation vials, and the radioactivity was determined. In the case of microsomes, bound and free [^{3}H]IP_4 were separated by filtration under vacuum employing GF/B Whatman filters, followed by rapid rinsing with 5 mL of ice-cold binding medium. The filters were transferred to 5-mL Biofluor liquid scintillation vials, and the radioactivity was determined. The binding on the cytosolic fraction was carried out as described here except that the receptor protein was precipitated with 18% poly(ethylene glycol). K_D and B_{max} were determined by Scatchard analysis of saturation isotherms at the optimum pH for each membrane fraction.

Marker Enzyme Activity. 5′-Nucleotidase activity was determined according to Kai et al. (1966) with minor modifications as described (Masmoudi et al., 1989). NADPH−cytochrome *c* reductase activity was determined as described (Crane & Löw, 1976). Cytochrome *c* oxidase was determined according to Smith (1955), and NAD pyrophosphorylase was determined according to Kornberg (1955).

RESULTS

Subcellular Fractions. The procedures for isolation of nuclei (Masmoudi et al., 1989) and other membrane fractions (Malviya et al., 1990) have been described earlier, and the purity of the fractions was verified by determining respective marker enzyme activities. A sucrose gradient centrifugation for the purification of plasma membrane was carried out, modifying the earlier protocol (Malviya et al., 1990) to ensure the Fraction's purity, which was duly attested by the presence of 5′-nucleotidase, a plasma membrane marker enzyme activity (Table 1).

Table 1: Marker Enzyme Activity in Rat Liver Membrane Fractions[a]

	homogenate		plasma membrane		microsomes		nuclei		mitochondria	
marker enzymes	sp act.	total act.	sp act.	total act.	sp act.	total act.	sp act.	total act.	sp act.	total act.
5′-Nucleotidase	4.1	20.5	32.0	3.52	0.04	0.02	0.21	0.04	0.36	0.2
NADPH cytochrome *c* reductase	7.0	35.0	0.36	0.04	19.8	9.7	2.0	0.42	4.4	2.45
cytochrome *c* oxidase	12.0	60.0	0.23	0.025	0.35	0.175	0.03	0.066	63.0	35.0
NAD pyrophosphorylase	4.0	20.0	0.18	0.02	0.41	0.2	24.2	5.08	0.18	1.0
Total protein (mg)	5000		110		490		210		555	

[a] Membrane fractions were prepared as described in Materials and Methods. Plasma membranes were purified by sucrose gradient centrifugation and were not contaminated by other membrane fractions as attested by marker enzyme activity. Nuclei were prepared by homogenizing the liver pieces in 1.3 M sucrose and maintaining 2.2 M sucrose in subsequent centrifugation steps. Specific activity (sp act.) is expressed as nmol/min/(mg of protein), and the total activity is expressed as μmol/min. These data are the mean of two independent subcellular fractionations with replicates varying less than 10%. Protein was determined according to Bradford (1976).

A comparative evaluation of other marker enzymes (Table 1) such as NADPH−cytochrome *c* reductase for microsomes, cytochrome *c* oxidase for mitochondria, and NAD pyrophosphorylase for the nuclei validates the purity of the various membrane fractions used in the studies reported in this paper.

Effect of pH on [^{3}H]IP_4 Binding. Figure 1a illustrates the total specific [^{3}H]IP_4 binding quantitatively distributed in various subcellular fractions originating from 5 g of liver homogenate. The binding described here as total binding is in fact [^{3}H]IP_4 specific binding considering total protein pertaining to each membrane fraction as mentioned in Table 1. The maximum total binding was seen at pH 7.0−7.5 in the homogenate (Figure 1a, upper plot). Total IP_4 binding in the nuclei occurred in the pH range 6.0−7.0, although binding at pH 6.5 was found to be optimal. Interestingly, at pH 6.5 almost negligible IP_4 binding was seen in plasma membranes or microsomal fractions. In the plasma membranes the IP_4 binding sites were seen in the pH range 7.0−8.0, with, pH 7.5 being the optimum pH for this membrane fraction. The optimum pH for microsomal IP_4 binding sites was 8.0. In fact, the binding sites in subcellular fractions appeared to be well represented in the whole homogenate. For instance, major binding in the homogenate was found in the pH range 7.0−7.5, which is the pH range over which nuclei, plasma membranes, and microsomes contribute cumulatively (Figure 1a). At pH 6.5 the total binding sites in the homogenate represent only the contribution from the nuclei. An analysis of the percentage of binding in subcellular fractions at various pH values, considering the homogenate binding as 100%, is depicted in Table 2. Nuclei appeared to be a major site for IP_4 binding, whereas IP_4 binding was almost negligible in mitochondria. IP_4 binding was poorly represented in microsomes. The majority of nuclear IP_4 binding occurred below pH 7.0, whereas the binding above pH 7.0 involved the plasma membranes or microsomes. About 15% of the total homogenate [^{3}H]IP_4 binding was seen in the rat liver cytosolic fraction (data not shown), reflecting the binding of IP_4 to inositol phosphate metabolic enzymes. In the cytosol the binding was evaluated at pH 7.0.

pH-Dependent Nature of [^{3}H]IP_4 Binding. The effect of pH on specific [^{3}H]IP_4 binding (per milligram of protein) in various membrane fractions is illustrated in Figure 1b. Each membrane fraction showed pH selectivity for specific binding. For instance, plasma membrane, nuclei, and microsomes

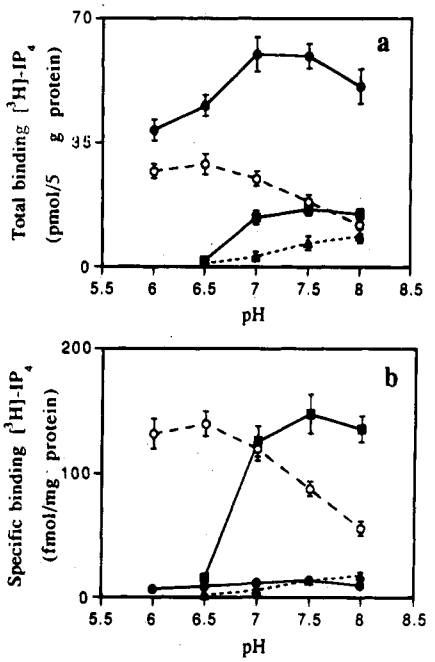

FIGURE 1: Effect of pH on (a) total and (b) specific [^{3}H]IP_4 binding in membrane fractions. [^{3}H]IP_4 binding was carried out as described in Materials and Methods. The concentration of radioligand was maintained at 2 nM, the specific activity being 21 μCi/nmol. For pH 6.0 and 6.5, 10 mM MES buffer was used. For pH 7.0, 7.5, and 8.0, 50 mM Tris-HCl was the buffer used. The amounts of protein per binding assay were as follows: plasma membrane, 0.4 mg; nuclei, 0.8 mg; microsomes, 1.0 mg; and homogenate, 1.0 mg. Nonspecific binding was determined by including 10 μM IP_4 in the binding assay. The total volume of the binding assay was maintained at 400 μL. Homogenate (●), plasma membranes (■), nuclei (○), and microsomes (▲). These experiments were carried out in quadruplicate. Total binding is specific IP_4 binding calculated in terms of total protein in each fraction as mentioned in Table 1. At optimum pH plasma membranes showed 3700 cpm specific and 500 cpm nonspecific binding; nuclei at their optimum pH had 3500 specific and 400 nonspecific cpm values, microsomes had 300 and 400 cpm specific and nonspecific binding, respectively, with replicates varying less than 10%. Total counts introduced in each assay was in the range of 20 000 cpm.

showed 7.5, 6.5, and 8.0 pH optima for maximum specific binding sites. The specific binding sites were pronounced in the plasma membranes and nuclei and were poorly represented in the microsomes. It did seem that the putative IP_4 receptor proteins were well distributed in the plasma membranes and the nuclei. The pH-dependent nature of the IP_4 binding sites in various membrane fractions was well

Table 2: Percentage of Total [^{3}H]IP_4 Binding in Subcellular Fractions, Taking Homogenate Binding as 100%[a]

pH	plasma membranes	nuclei	microsomes	mitochondria
6.0	ND	70	ND	ND
6.5	4	64	2	1
7.0	23	41	5	2
7.5	27	31	12	2
8.0	29	23	18	1

[a] Membrane fractions were prepared as described in Materials and Methods. The concentration of [^{3}H]IP_4 was 2 nM. This concentration was chosen to reach the K_D values determined by the Scatchard plot of membrane fractions. The binding was carried out at 0 °C on ice for 20 min. The amounts of protein per binding assay in plasma membranes, nuclei, microsomes, and mitochondria were 0.4, 0.8, 1.0, and 1.5 mg, respectively. ND = not detected. Cytosol contained 15% of the total homogenate binding at pH 7.0.

founded as evaluated here (Figure 1). In the plasma membranes and nuclei the pH effect on IP_4 binding did not result due to the use of a different buffer. These results were verified using Bis−Tris and MES for pH 6.5 and HEPES and Tris-HCl for pH 7.5. Almost identical [^{3}H]IP_4 binding was observed irrespective of the buffer employed at a particular pH (data not shown).

[^{3}H]IP_4 Binding Selectivity. Inositol 1,3,4,5-tetrakis([^{3}H]-phosphate) binding sites in the nuclei, plasma membranes, and microsomes were examined by competitive displacement of the radioligand with IP_4, Ins(3,4,5,6), IP_5, and IP_3 as illustrated by Figure 2. Maximum inhibition of binding was observed with IP_4 in all three subcellular fractions examined, i.e., plasma membranes (Figure 2a), nuclei (Figure 2b), and microsomes (Figure 2c). Almost identical rank orders of inhibition were found in the three membrane fractions: IP_5, Ins(3,4,5,6), and IP_3. The competitive displacement with IP_4 revealed two binding sites in the nuclei as compared with a single binding sites in the plasma membranes and in the microsomes (Figure 2). These experiments were carried out by choosing the optimum pH for each membrane fraction.

Scatchard analysis of saturation isotherms was carried out with progressively increasing [^{3}H]IP_4 concentrations. The Scatchard analysis (Figure 3) was performed at the optimum pH for each membrane fraction, i.e., at pH 7.5, 6.0, and 8.0 for plasma membranes, nuclei, and microsomes, respectively. Two distinct binding sites were characterized in the nuclei (Figure 3b), whereas plasma membranes (Figure 3a) and microsomes (Figure 3c) showed, apparently, single binding site characteristics. However, the curves for displacement of [^{3}H]IP_4 by IP_4 (Figure 2) span about 5 orders of magnitude, indicating the presence of minor subpopulations of IP_4 binding sites in these fractions. The K_D and B_{max} values for various subcellular fractions derived from graphic analysis of Scatchard plots are depicted in Table 3.

Comparative Displacement with IP_4 and IP_6 of [^{3}H]IP_4 Binding to the Plasma Membrane. Since IP_4 and IP_6 receptor proteins have been shown to be closely associated with rat cerebellar membranes (Theibert et al., 1991), it was considered necessary to examine the [^{3}H]IP_4 binding in the rat liver plasma membrane as displaced by these two inositol phosphates (Table 4). IP_4 displaced the radioligand with a distinct IC_{50} (3.0 ± 0.5 nM) as compared with the IC_{50} obtained for IP_6 (120.0 ± 15 nM).

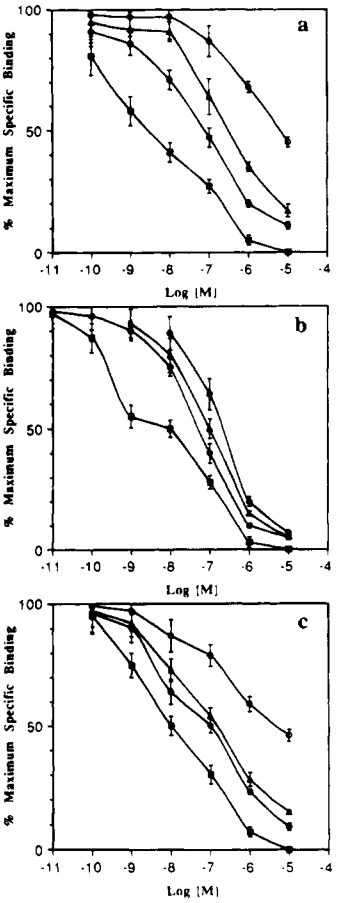

FIGURE 2: Displacement of [^{3}H]IP_4 by various inositol phosphates in plasma membranes (a), nuclei (b), and microsomes (c). The radioligand binding was displaced by employing various concentrations of IP_4 (■), IP_5 (●), Ins (3,4,5,6) (▲), and IP_3 (○). The optimum pHs for binding in various membrane fractions were used: plasma membranes, pH 7.5; nuclei, pH 6.5; microsomes, pH 8.0. The binding assay medium contained 1 mM EDTA, 100 mM KCl, 20 mM NaCl, and 10 mM MES, pH 6.5, in the case of nuclei; MES was replaced by 50 mM Tris-HCl, pH 7.5 in the case of plasma membranes and pH 8.0 in the case of microsomes. Each binding assay contained proteins from plasma membranes (0.4 mg), nuclei (1.0 mg), and microsomes (1.0 mg). The concentration of [^{3}H]IP_4 was 1 nM in the case of plasma membranes and microsomes and 2 nM in the case of nuclei, the specific activity being 21 μCi/nmol. The details of the binding protocols are described in Materials and Methods.

DISCUSSION

It is through the characterization of specific binding sites that insight into the inositol phosphate second messenger function has been deciphered. Out of various inositol phosphates, the inositol 1,4,5-tris(phosphate) receptor is the best characterized as a receptor protein, and its pivotal role in intracellular calcium regulation (Ferris & Snyder, 1992) is very well established. Inositol 1,3,4,5−tetrakis(phosphate) binding sites have been characterized in a number of tissues (Bradford & Irvine, 1987; Enyedi & Williams, 1988; Enyedi et al., 1989; Challiss et al., 1991; Donié & Reiser, 1989) and have been demonstrated to possess divergent pH requirements. Multiple molecular masses have been assigned to the IP_4 receptor protein. IP_4 binding sites are fewer in number in peripheral tissues as compared with the cerebellum. To the best of our knowledge subcellular locations of IP_4 binding sites in a peripheral tissue have not been examined earlier. We have documented (Köppler et al., 1993) the inositol phosphate selectivity of IP_4 binding in

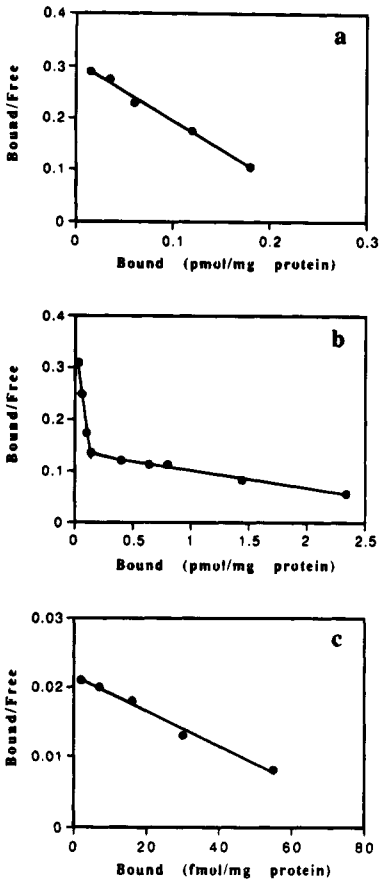

FIGURE 3: Scatchard plots of [^{3}H]IP_4 binding to plasma membranes (a), nuclei (b), and microsomes (c). Scatchard analysis was carried out by utilizing progressively increasing concentrations of [^{3}H]IP_4 from 0.1 to 100 nM in the case of nuclei and from 0.1 to 10 nM in the case of plasma membranes and microsomes. The buffer system for each membrane fraction and the binding protocols are described in Materials and Methods. Experiments were carried out on two independent preparations of various membranes in quadruplicate. K_D and B_{max} values are tabulated in Table 3.

Table 3: Characteristics of IP_4 Binding Sites in Membrane Fractions at Their Respective Optimum pH[a]

	plasma membranes	nuclei	microsomes
pH	7.5	6.5	8.0
K_D (nM)	0.8 ± 0.15	K_{D1} = 1.6 ± 0.15 K_{D2} = 57 ± 6.0	9.0 ± 0.8
B_{max} [fmol/(mg of protein)]	250 ± 20	B_{max1} = 250 ± 20 B_{max2} = 3700 ± 400	80.0 ± 7.0

[a] K_D and B_{max} were determined by Scatchard analysis (Figure 3) of saturation isotherms as described in Materials and Methods. In the case of plasma membranes and nuclei, each binding assay contained 0.4 and 0.8 mg of protein, respectively. In the case of microsomes, 1 mg per assay was the amount of protein.

purified rat liver nuclei. This study has prompted us to undertake a rigorous look into the IP_4 binding characteristics in various subcellular fractions derived from the rat liver. The metabolism of IP_3 and IP_4 has been studied earlier in rat liver (Hansen et al., 1986).

Substantial IP_4 binding in the liver homogenate was manifested in the pH range from 6.5 to 8.0 (Figure 1a). This made it necessary to characterize IP_4 binding in the plasma membranes, nuclei, and microsomes. A pH-dependent nature of IP_4 binding sites has emerged in various subcellular compartments (Figure 1a). Interestingly enough, if one needs

Table 4: Comparative Evaluation of [^{3}H]IP_4 Displacement with IP_4 and IP_6 in the Plasma Membrane[a]

inositol phosphates	% of specific bound	IC_{50} (nM)
IP_4		3.0 ± 0.5
1 nM	80	
10 nM	40	
100 nM	27	
1 μM	05	
IP_6		120 ± 15.0
1 nM	95	
10 nM	80	
100 nM	55	
1 μM	30	

[a] The binding was carried out at pH 7.5, maintaining the radioligand concentration at 1 nM (sp act., 21 μCi/nmol) as described in Materials and Methods. These data are replicates of three experiments for which standard errors were less than 10%.

to distinguish between nuclear and nonnuclear IP_4 binding in the rat liver, the binding represented at acidic pH (6.5) can be taken as the binding contributed solely by the nucleus. As compared with plasma membranes or nuclei, microsomes did not seem to possess a large number of binding sites (Table 2). Looking at the distribution of proteins (Table 1) in the various subcellular fractions, microsomes did have a substantial share, and therefore one would have expected a proportionate share of IP_4 binding sites in this organelle. But this is not the case. The rationale for poor IP_4 binding in microsomes is provided by the Scatchard analysis, revealing a low B_{max} as compared with that of plasma membranes or nuclei. In spite of a K_D of 9.0 nM, the density of IP_4 binding sites appeared poor in the microsomes. So far as the plasma membranes and nuclei were concerned, a high density of binding sites was found. A large number of putative IP_4 receptor proteins in a lesser amount of protein representing plasma membranes or nuclei as compared with microsomes attests the importance of the role of IP_4 in the case of these two membrane fractions. Plasma membranes (Wilcox et al., 1993) and nuclei (Malviya, 1994) are supposed to be concerned with calcium fluxes mediated by IP_4.

Furthermore, the inositol phosphate selectivity of IP_4 binding sites was established by the specific displacement of [^{3}H]IP_4 with various inositol phosphate derivatives (Figure 2). IP_4 inhibited most strongly the radioligand binding to the various membrane fractions. Almost identical patterns of rank order inhibition with IP_5, Ins(3,4,5,6), and IP_3 were observed in plasma membranes, nuclei, and microsomes. This showed that the binding sites under study were similar in all three membrane fractions.

The properties of the binding sites were further revealed by Scatchard analysis (Figure 3) of the saturation isotherm, which is a more precise evaluation of ligand binding characteristics. In terms of K_D the plasma membranes (0.8 nM) and nuclei (1.6 nM) appeared to exhibit powerful affinity for IP_4, whereas the microsomal fraction showed comparatively weak affinity (K_D = 9.0 nM), although a single binding site was apparent in the plasma membranes and microsomes (Table 3). This notwithstanding, the displacement curves (Figure 2) seem to show certain subpopulations of minor binding sites, which may be attributed to multiple IP_4 receptor proteins as described earlier (Donié & Reiser, 1991; Theibert et al., 1991). Nuclei contained high-affinity (K_{D1} = 1.6 nM) and low-affinity (K_{D2} = 57 nM) binding

sites. These data on the binding sites favor that the characteristics of IP_4 receptor in the three membrane compartments are not the same. Such thinking found further support from the data on density of IP_4 receptor proteins distributed in the three membrane compartments as evaluated by B_{max}. The density of IP_4 receptors in the plasma membranes was identical to that of the high-affinity sites of the nucleus [B_{max} = 250 fmol/(mg protein)], suggesting thereby that IP_4 may have a similar function in these two membrane fractions. Indeed, microsomes are the site for calcium storage, whereas plasma membranes or nuclei are well known not to serve as calcium pools, but to subserve gating mechanisms for calcium fluxes. It is indeed interesting to observe that the plasma membrane IP_4 receptor is not essentially different from the nuclear high-affinity receptor if one considers either the K_D or the B_{max}, which duly attest to the role of the IP_4 receptor in calcium fluxes from extracellular compartments in the case of plasma membranes and the rise of intracellular calcium mediating calcium entry into the nucleus (Köppler et al., 1993) in the presence of IP_4.

A number of inositol phosphate binding proteins have been purified from rat cerebellar membranes (Theibert et al., 1991). Certain common binding sites for IP_4 and IP_6 have been reported in one of the proteins purified from rat cerebellum and IP_6 has been shown to affect calcium accumulation in cultured cells (Nicoletti et al., 1989). Furthermore, it has been shown (Timerman et al., 1992) that the cerebellar membrane protein that binds to IP_6 and IP_4 is identical to the corresponding sequence of the clathrin assembly protein AP-2 (also referred to as clathrin adaptor or associated protein). Therefore, the question whether rat liver plasma membrane [^{3}H]IP_4 binding sites could be displaced with IP_6 was addressed (Table 4). The IC_{50} of IP_6 was about 40-fold higher than the IC_{50} of IP_4 in the rat liver plasma membrane. This tempts us to suggest that the IP_4 binding protein in the liver plasma membrane is a distinct IP_4 receptor protein from the IP_6 receptor and is not the clathrin assembly protein AP-2 (Voglmaier et al., 1992).

The plasma membrane IP_4 binding site that we are reporting is not due to contribution from the liver plasma membrane Ins(1,3,4,5)P_4 5-phosphatase (Shears et al., 1987). In this regard we may point out that Ins(3,4,5,6)P_4, Ins(1,3,4,5,6)P_5, and Ins(1,2,3,4,5,6)P_6 are very poor inhibitors of Ins(1,3,4,5)P_4 5-phosphatase (Hughes & Shears, 1990), and therefore it would be expected that the affinity for Ins(1,4,5)P_3 should exceed that for other inositol phosphates. In the studies reported here this is not the case. In fact the K_D for IP_3 is largely higher (Figure 2a and Table 3) than the K_D for other inositol phosphates. Thus it may be safely argued that the IP_4 binding site seen in the liver plasma membrane is the Ins(1,3,4,5)P_4 receptor protein.

At this stage there is no unanimous opinion regarding the role of IP_4 in the cellular response, although it is generally agreed that cellular calcium mobilization is regulated by IP_4. Conflicting reports are documented on the mode of action of IP_4. For example, it is suggested that IP_4 exerts its influence by binding to a protein (Pietri et al., 1990), that there is a synergy between the action of IP_3 and IP_4 (Gawler et al., 1991a), that the order of addition between IP_3 and IP_4 determines the action of IP_4 (Hill & Boynton, 1990), that IP_4 enhances the action of IP_3 (Spat et al., 1987), that IP_4 directly releases calcium (Gawler et al., 1991b), that the intracellular calcium level determines IP_4-mediated calcium influx (Pitlet et al., 1989), and that nuclear calcium uptake is mediated by an ATP-independent IP_4 pathway (Köppler et al., 1993). Such a diversity of responses mediated by IP_4 indicates multiple ways by which IP_4 receptor is capable of being regulated. According to the data reported here there is a multiplicity of pH responses at various cellular locations. We must know which of the IP_4-mediated functions enunciated above is carried out by which type of cellular membrane to fully comprehend the cellular role of IP_4. In this background we are tempted to suggest that the role of IP_4 mediated by its receptors located in various membrane fractions in a cell can be regulated by intracellular pH modulation. Changes in intracellular calcium levels linked with changes in intracellular pH during growth promotion are well studied (Cassel et al., 1983; Moolenar et al., 1984; Rozengurt, 1986; Johnson et al., 1976; Short et al., 1993). The data presented in this paper provide interesting clues to the interrelationship between cellular pH response, as evidenced by the pH sensitivity of IP_4R, and calcium fluxes, which are, undoubtedly, regulated by IP_4 (Wilcox et al., 1993; Malviya, 1994).

Distinct patterns of distribution of IP_4 receptors in different organelles in the same tissue may be relevant to the organelle-specific function of IP_4. However, purification and characterization of IP_4 receptor proteins from various membrane fractions will adequately address the role played by IP_4. We are currently purifying the IP_4 receptor from rat liver nuclei since our primary concern is to understand nuclear events (Köppler et al., 1993; Matter et al., 1993; Rogue et al., 1990, 1993; Block et al., 1992; Malviya & Block, 1993; Rogue & Malviya, 1994) during signal transduction.

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