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CHANGES IN 1,4,5-INOSITOL TRISPHOSPHATE BINDING FOLLOWING PARTIAL HEPATECTOMY

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SUMMARY: Hepatic parenchymal cells possess two receptors for 1,4,5-trisphosphate, one isolated with the plasma membrane fraction and another isolated with the nuclear fraction. Their interaction with antibodies generated against the receptor in the cerebellum indicates that these two receptor proteins are different. The potential involvement of the nuclear receptor in rapid cell proliferation was tested by measuring [^{3}H]-IP_3 binding, following partial hepatectomy. In nuclear fractions isolated 18 hours after the operation, a 33% decline in binding sites was detected. In nuclear fractions isolated 30 hours after the operation, a 60% decline in the binding sites was detected, but the K_d remained unchanged. A 70% decrease in binding sites was also detected in the plasma membrane fraction. These results show that partial hepatectomy is associated with a parallel loss of receptor sites in the nuclear and plasma membrane fractions. © 1994 Academic Press, Inc.

Stimulation of the liver by several hormones leads to the generation of inositol IP_3 and subsequently to increases in cytosolic free Ca^{2+} levels (1). The initial step in the mode of action of IP_3 is binding to a specific receptor protein. In the liver, two different IP_3 binding sites have been identified, one isolated with a plasma membrane fraction and another with the nuclear fraction (2,3). The increases in cytosolic free Ca^{2+} level following hormone administration can be attributed to the binding of IP_3 to the receptor which is isolated with the plasma membrane fraction in the liver and in several other cell types (4). The function of the IP_3 receptor which is localized in the nuclear fraction is not known (5).

The possibility that IP_3 has a role in nuclear function is indicated by the presence of enzymes synthesizing phosphoinositides in the nucleus (6,7). Thus, the nuclei might have a separate, autonomous regulatory mechanism to generate IP_3. This nuclear IP_3 generating system might function independently and contribute to the regulation of intranuclear Ca^{2+} concentrations (8). That intranuclear Ca^{2+} concentrations might be regulated independently of the cytosolic Ca^{2+}

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ABBREVIATION: IP_3, Inositol 1,4,5-trisphosphate.

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is unexpected in light of the presence of the nuclear pores in the nuclear membrane, because the pore structure suggests a potential equilibration between cytosolic and nuclear Ca^{2+}. However, the presence of a distinct Ca^{2+} gradient between the cytoplasm and the nucleus has been demonstrated in liver cells (9). Ca^{2+} uptake into the nucleus was shown to be ATP-dependent (10). The Ca^{2+}-ATPase which carries out this transport was demonstrated to be identical to the one present in the endoplasmic reticulum (11). A release of the accumulated Ca^{2+} upon the addition of IP_3 to isolated nuclear fraction was also observed (12), a finding which supports the notion that the nuclear IP_3 generating system is involved in the regulation of nuclear Ca^{2+} levels and hereby might modulate nuclear function (13).

If the nuclear phosphoinositide-specific phospholipase C and the changing IP_3 levels are involved in modulating nuclear Ca^{2+} levels, several important questions need to be clarified. Thus, what agents affect nuclear IP_3 production? Are those agents the same or different than those which alter plasma membrane IP_3 generation? Are the number and affinity for IP_3 of the nuclear receptors changing in various physiological conditions and if yes, do these changes occur in parallel with the changes in the plasma membrane IP_3 receptor or independently of it?

In order to answer some of the questions on the function of the nuclear IP_3 receptor, the partially hepatectomized rat was employed as a model. Partial hepatectomy in the rat is followed by liver regeneration, which is a sharp burst of proliferative activity that ceases when the original mass has been restored. Rat liver regeneration provides a well characterized model in which the processes of cell proliferation are studied (14).

Recent studies demonstrated that following partial hepatectomy, changes occur in the regenerating rat liver which indicate the possibility that nuclear Ca^{2+} sequestration is altered: A three-fold incease in calmodulin was reported in liver nuclei 24 hours after partial hepatectomy (15). Increases in α-spectrin, actin and a caldesmon-like protein were also observed (16). Data which indicate possible changes in nuclear calcium levels during cell proliferation were also obtained by Cocco et al. (13, 17), who reported changes in nuclear phosphoinositide content in various stages of cell division. Also, an increase in the activity of phosphoinositide-specific phospholipase C in rat liver nuclei prepared from regenerating rat livers was recently reported. This increase occurred parallel to the increases observed in DNA polymerase α activity (7). The regenerating rat liver was therefore chosen as a model to tell whether or not changes in IP_3 binding occur during cell proliferation and if so, whether the changes occur parallel in the plasma membrane and nuclear fraction.

MATERIALS AND METHODS

Animals. Male, Sprague-Dawley rats, weighing 130-180 g, were used in all the experiments.

Operational techniques. Partial hepatectomy was performed under light ether anesthesia in sterile conditions. Two-thirds of the liver was removed. Control rats were sham-operated. Either 18 or 30 hours after the operation, livers were removed for preparation of subcellular fractions.

Preparation of subcellular fractions. Plasma membrane fractions were prepared by a modification of Ray's procedure as previously described (18). Nuclei were prepared by the method of Nicotera et al. (12).

Measurement of [^{3}H]-IP$_3$ binding. Binding of IP_3 to the different fractions was carried out as described by Feng et al. (18). In short, membranes or nuclei, (lmg/ml) were incubated at O °C in a medium containing 25 mM Na_2HPO_4, pH 7.4, 100 mM KCL, 20 mM NaCL, 1 mM EDTA 0.1% bovine serum albumin and 5 nM [^{3}H]-IP$_3$ (21 Ci/mmol). Non-specific binding was determined in the presence of 5 nM IP_3. Non-bound [^{3}H]-IP$_3$ was removed by the millipore technique, and the radioactivity retained on the filter was determined by the scintillation method.

Western blot analysis of proteins. For dot-blotting of proteins, the protein samples were dissolved in SDS and applied to nitrocellulose papers. The dots were incubated for 30 minutes in TWEEN 20-phosphate-buffered saline (TPBS, 0.05% TWEEN-20 in PBS) and then incubated with primary antibody (Rabbit anti-IP$_3$R antibody, 1:1000 dilution in TPBS containing 0.1% BSA and 0.1% NaN_3) for 2 hours at room temperature on a rocker apparatus. After washing three times with TPBS, the blots were incubated with secondary antibody (Goat anti-rabbit IgG, horseradish peroxidase conjugate, 1:1000 dilution in TPBS containing 0.1% BSA) for 1 hour. The blots were washed three times with PBS and incubated in a color development reagent (10 ml PBS, 2 ml methanol containing 0.3% 4-chloro-1-naphthol and 8 ul of 30% H_2O_2) for about 10 minutes. Subsequent washes were in distilled H_2O. For western-blot analysis, the samples were dissolved in SDS and applied to an SDS-PAGE gel as in previous experiments (3). The stacking gel was 3% and the separating gel 6%. Subsequently, the proteins were transferred to mitrocellulose paper and treated as described above with the exception that the dilution of the primary antibody was 1:500. The antibody was a gift from Dr. F. A. Lai.

Protein determination. Protein content was determined by the method of Hill and Straka (19). This method allows protein determination in the presence of DTT.

Statistical analysis. Data were analyzed by the paired t-test using a computer program (Primer Biostatistics: The Program).

RESULTS AND DISCUSSION

The binding of [^{3}H]-IP$_3$ to liver fractions isolated from normal rat livers is illustrated in Figure 1. In the presence of 5nM IP_3, the binding of IP_3 to the plasma membrane and nuclear fractions showed similar characteristics. The magnitude of binding was in the same range, about 60 fmol IP$_3$/mg protein, and heparin inhibited binding in both fractions.

In spite of these similarities in binding characteristics, the receptors present in these two cell fractions are not identical. A dot-blot, illustrating the interaction between antibodies generated against the receptor in the cerebellum with the two liver fractions, is shown in Figure 2 A. The plasma membrane fraction does not interact with the antibodies. Antibodies prepared against the brain receptor in another laboratory (T. Sudhof - results not shown) gave similar results. In Figure 2 B., western-blot analysis of the same proteins illustrate that the antibodies interact with a protein present in the cerebellum, which has a molecular weight corresponding to the IP_3 receptor. These data indicate that the receptor present in the plasma membrane is

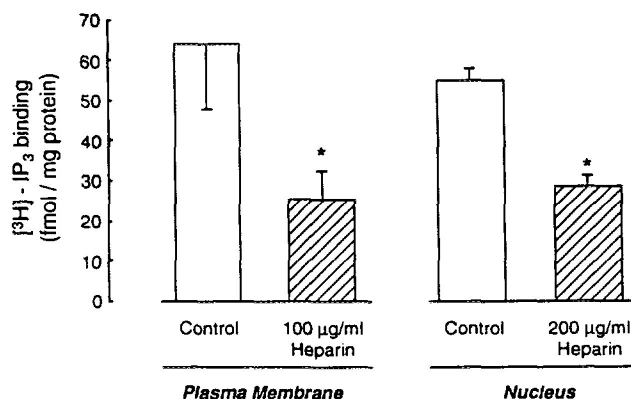

Figure 1. **Effect of heparin on the binding of [^{3}H]-IP$_3$ to the plasma membrane and nucleus.** Binding of [^{3}H]-IP$_3$ was assayed as described in **Materials and Methods**. The concentration of [^{3}H]-IP$_3$ was 5 nM. * indicates significant difference between the experiment and the control. Data were Mean $\pm$ SEM of 4 experiments.

different from that isolated from the cerebellum. In contrast, the receptor present in the nuclear fraction does react with antibodies prepared against the brain receptor (Figure 2). An interaction between antibodies generated against the purified brain receptor and the nuclear receptor has been noted previously by Matter et al. (2). Thus, the receptor present in the nuclear fraction seems to be identical to the isoform present in the cerebellum, while the one present in the plasma membrane fraction is not. According to these data, the liver possesses two different isoforms of the IP$_3$ receptor, and each isoform is sequestered into a separate subcellular organelle.

In order to evaluate whether or not rapid cell proliferation is associated with changes in characteristics of the nuclear IP$_3$ receptor, binding studies were carried out. In comparison, binding was studied also in the plasma membrane fraction. In the first set of experiments liver fractions were prepared 18-20 hours following partial hepatectomy. This time was chosen because following partial hepatectomy many of the changes which occur are phasic and some of the changes are most pronounced at that time range. For instance, a marked increase in nuclear diacylglycerol which was maximal around 20 hours post operation was observed by Banfic et al. (20). This was associated with the specific translocation of protein kinase C to the nucleus (20). The observed increases in nuclear calmodulin were also maximal at 24h (15). In another study, this period was characterized by the largest percent of hepatocytes with [^{3}H]-thymidine labelled nuclei, indicating increased DNA sythetic activities (21).

Binding of [^{3}H]-IP$_3$ to isolated liver nuclei, measured under the same experimental conditions as in Figure 1 with the exception that nuclei from 18 hours post-hepatectomized rats were used, showed a decrease in binding; sham operated: 19$\pm$ 2 fmol/mg protein; hepatectomy:

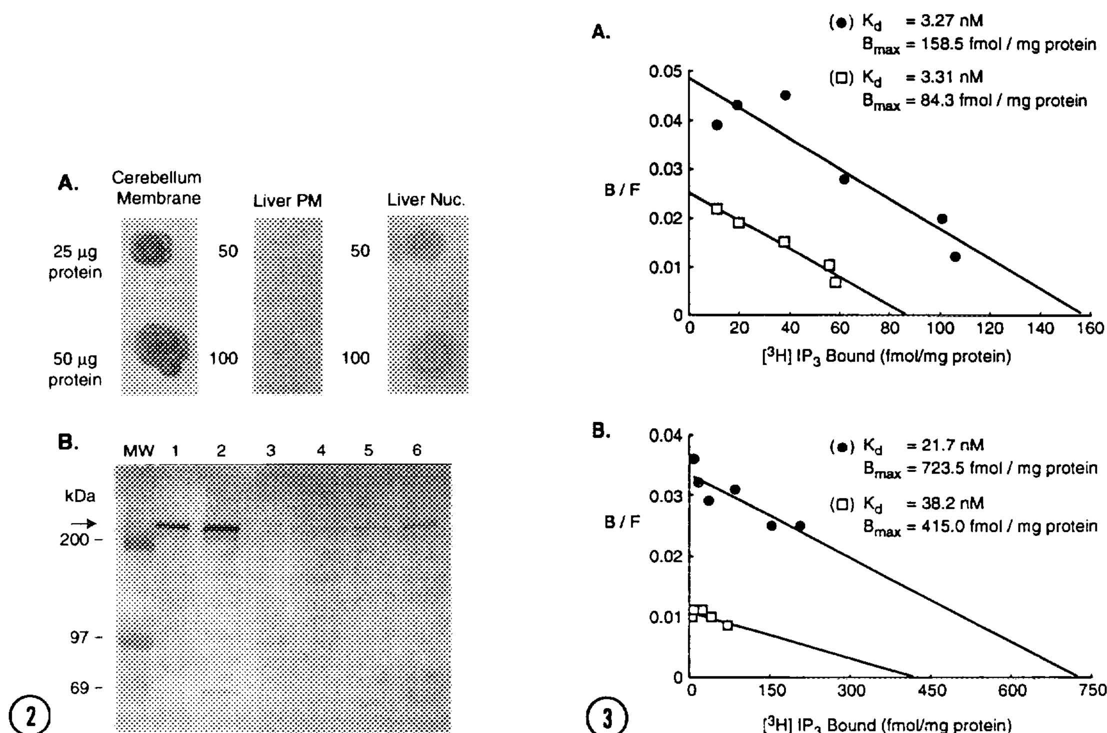

Figure 2. **Western blot analysis for IP_3 receptor.** The dot blot was performed as described in **Materials and Methods (Fig. 2A.)**. The amounts of protein were shown on the **Fig.** Lanes: 1. Cerebellum membrane, as control; 2. Liver plasma membrane; 3. Liver nucleus. In Western blot analysis, the same amounts of proteins were applied as in the dot-blot **(Fig. 2B.)**.

Figure 3. **Scatchard plot for $[^3H]$-IP_3 binding to plasma membrane and nucleus.** $[^3H]$-IP_3 binding was carried out as described in **Materials and Methods**. (●) control (□) hepatectomy. A: Nucleus; B: Plasma membrane.

13 ± 1 fmol/mg protein, 33% decrease. A similiar decrease in $[^3H]$-IP_3 binding was observed also in the plasma membrane fraction, sham operated: 91 ± 4 fmol/mg protein; hepatectomy: 62 ± 10 fmol/mg protein, a 31% loss. Both results were statistically significant, $p = 0.03$.

In order to examine the time dependence of these changes, similar measurements were carried out in both fractions 30 hours following partial hepatectomy. In these experiments, the K_d and B_{max} were also determined. The results show a further decrease in $[^3H]$-IP_3 binding in both fractions (Figure 3). The K_d in the nuclear fractions was unaffected by the operation. In the plasma membrane preparation the K_d was increased in the sham-operated rats over values which we previously found (18). This might indicate that the anesthesia and trauma associated with the operation affects the plasma membrane IP_3 receptor. However, the decrease observed in the hepatectomized rats was significant over the sham-operated animals. This indicates that partial

hepatectomy evokes changes either directly in the IP_3 receptor number and binding characteristics, or that the plasma membrane composition changes in a way that IP_3 binding decreases. Huerta-Bahena et al. (22) reported that partial hepatectomy does not reduce phosphatidylinositol labeling following vasopressin stimulation, though all the other Ca^{2+}-dependent metabolic responses were inhibited by the operation. The present observation that partial hepatectomy decreases $[^3H]$-IP_3 binding and decreases the affinity of the receptor for its ligand can explain, at least partially, the lack of metabolic responses to vasopressin and angiotensin II in partially hepatectomized rats reported by these authors.

The consequences of the loss of $[^3H]$-IP_3 binding in the nuclear fraction is difficult to interpret because of the limited knowledge of the role that phosphoinositides play in nuclear functions. Cocco et al (13,17) reported a decrease in phosphatidylinositol bisphosphate synthesis in nuclei of growing cells or in cells treated with mitogenic growth factions. The decrease in IP_3 binding observed in the present study is also pointing towards a decrease in phosphoinositide utilization during rapid cell growth. Whether these changes in IP_3 binding are associated with changes in the nuclear Ca^{2+} levels or not is not known and needs to be examined. The dramatic decrease in nuclear IP_3 binding observed in the present studies supports the notion proposed by Cocco et al (13) that phosphoinositides made inside the nucleus might have a role in the process of cell division.

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