

Mechanism of nuclear calcium signaling by inositol 1,4,5-trisphosphate produced in the nucleus, nuclear located protein kinase C and cyclic AMP-dependent protein kinase

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1. ABSTRACT

Nuclear phospholipase C-gamma 1 can be phosphorylated by nuclear membrane located epidermal growth factor receptor sequel to epidermal growth factor-mediated signaling to the nucleus. The function of mouse liver phospholipase C-gamma 1 is attributed to a 120 kDa protein fragment which has been found to be a proteolytic product of the 150 kDa native nuclear enzyme. The tyrosine-phosphorylated 120 kDa protein band interacts with activated EGFR, binds phosphatidyl-3-OH kinase enhancer, and activates nuclear phosphatidylinositol-3-OH-kinase, and is capable of generating diacylglycerol in response to the epidermal growth factor signal to the nucleus *in vivo*. Thus a mechanism for nuclear production of inositol-1,4,5-trisphosphate is unraveled. Nuclear generated inositol-1,4,5-trisphosphate interacts with the inner membrane located inositol-1,4,5-trisphosphate receptor and sequesters calcium into the nucleoplasm. Nuclear inositol-1,4,5-trisphosphate receptor is phosphorylated by native nuclear protein kinase C which enhances the receptor-ligand interaction. Nuclear calcium-ATPase and inositol-1,3,4,5-tetrakisphosphate receptor are located on the outer nuclear membrane, thus facilitating calcium transport into the nuclear envelope lumen either by ATP or inositol-1,3,4,5-tetrakisphosphate depending upon the external free calcium concentrations. Nuclear calcium ATPase is phosphorylated by cyclic AMP-dependent protein kinase with enhanced calcium pumping activity. A holistic picture emerges here where tyrosine phosphorylation compliments serine phosphorylation of key moieties regulating nuclear calcium signaling. Evidence are forwarded in favor of proteolysis having a profound implications in nuclear calcium homeostasis in particular and signal transduction in general.

2. INTRODUCTION

The discovery that inositol 1,4,5-trisphosphate (IP₃) mobilizes calcium from the ⁴⁵Ca²⁺ loaded isolated rat liver nuclei (1, 2) and that the inositol 1,4,5-trisphosphate receptor (IP₃R) is located on the nuclear membrane (1) established for the first time a signal transduction role of both inositol phosphate (3) and calcium (4, 5) at the level of the cell nucleus. An earlier discovery that IP₃ is an intracellular Ca²⁺-releasing messenger in permeabilized pancreatic acinar cells (6) provided a biological function to the IP₃, seeking a physiological role since its serendipitous discovery in 1953 (7). The elucidation of the primary structure of the IP₃R (8) and that it functions both as a calcium channel and a specific IP₃-binding protein universally located in all mammalian systems (9) laid the foundation for understanding the mechanism of IP₃-mediated cellular function. It is now well founded that nuclear calcium signaling is independently regulated from its cytosolic counterpart (10, 11). Autonomous nuclear calcium signaling pathways find support from the nuclear location of enzymes (12, 13) and phosphoinositides (14, 15, 16) involved in IP₃ generation.

Phospholipase C (PLC) catalyzes phosphatidylinositol-4,5-bisphosphate (PIP₂) hydrolysis to generate IP₃ and diacylglycerol (DAG). Several isoforms of PLC have been identified in the nucleus and the nuclear beta isozyme is characterized in some detail (3). The role of PLC-gamma 1 in the nucleus has also been investigated which, upon stimulation, associates with phosphatidylinositol-3-OH-kinase enhancer (PIKE), a nuclear GTPase (12) and consequently activates nuclear (17) phosphatidylinositol-3-OH-kinase (PI(3)K) activity. PIKE may be a widespread regulator of nuclear PI(3)K activity. *In vivo* studies, in mouse liver nuclei, revealed that epidermal growth factor (EGF) signaling

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Table 1. [³²P]IP₃ binding and microsomal marker enzyme activity

Fraction derived from rat liver	Protein, mg	[³² P]IP ₃ binding		NADH cytochrome c reductase activity		NADPH cytochrome c reductase (insensitive to antimycin A) activity	
		Specific fmol per mg of protein per min	Total pmol per min	Specific μmol per mg of protein per min	Total μmol per min	Specific μmol per mg of protein per min	Total μmol per min
Homogenate	4100 ± 205.0 (100)	80 ± 4.0	328 ± 16.4 (100)	0.008 ± 0.00040	32.8 ± 1.640 (100)	0.42 ± 0.0200	1723 ± 86.0 (100)
Nuclei	250 ± 12.5 (6.1)	87 ± 4.3	22 ± 1.1 (6.7)	0.0014 ± 0.00007	0.34 ± 0.017 (1.0)	0.025 ± 0.0012	6.25 ± 0.3 (0.4)
Final postnuclear material	337 ± 16.5 (8.2)	104 ± 5.0	35 ± 1.7 (10.7)	0.012 ± 0.00060	4.1 ± 0.197 (12.4)	0.41 ± 0.0200	138 ± 6.9 (8.0)
Homogenate with added microsomes*	4480 ± 223.5 (100)	87 ± 4.3	390 ± 19.5 (100)	0.012 ± 0.00060	53.7 ± 2.580 (100)	0.50 ± 0.0250	2240 ± 112.0 (100)
Nuclei	296 ± 14.7 (6.6)	85 ± 4.2	25 ± 1.2 (6.4)	0.0017 ± 0.00008	0.49 ± 0.024 (0.9)	0.03 ± 0.0015	8.8 ± 0.4 (0.4)
Final postnuclear material	324 ± 16.0 (7.1)	210 ± 10.5	68 ± 3.4 (17.4)	0.035 ± 0.00175	11.3 ± 0.528 (21)	0.95 ± 0.0465	309 ± 15.4 (13.8)

Total protein in the homogenate was 4102 mg. Total microsomal protein was 380 mg. [³²P]IP₃ specific and total binding were 158 fmol per mg of protein per min and 60 pmol per min, respectively; NADPH cytochrome c reductase specific and total activity were 0.06 μmol per mg of protein per min and 22.8 μmol per min, respectively; antimycin A-insensitive NADH cytochrome c reductase specific and total activity were 1.45 μmol per mg of protein per min and 551 μmol per min, respectively. Livers were homogenized in 1.3 M sucrose medium and the homogenate was centrifuged at 1000 xg. The resulting pellet was suspended in the same medium and was adjusted to 2.2 M sucrose by adding 2.4 M sucrose medium, followed by centrifugation at 100,000 x g for 1 hr. Nuclei were sedimented, whereas other cytoplasmic material, including contaminating microsomes, stayed above sucrose in the tube (this is termed final postnuclear material). The final concentration of [³²P]IP₃ was 0.5 nmol in the binding assay. The radioligand was displaced by 10 μM IP₃. The reduction of cytochrome c was monitored at 550 nm and the reductase activity was determined by using an extinction coefficient of 21.1 mM⁻¹·cm⁻¹. Antimycin A concentration was 5 mM. These data are mean ± SEM of two independent preparations. Numbers in parentheses represent percentage.

activates nuclear PLC-gamma 1 and generates DAG (18). The activated PLC-gamma 1 binds nuclear located (19) epidermal growth factor receptor (EGFR), thus an independent mechanism of IP₃ generation in the nucleus, divorced from the cytosolic events, is established (18).

The D3-phosphorylated inositol 3,4,5-trisphosphate (3,4,5-IP₃), which is a product of PI(3)K, is also present in the nucleus (20) and serves as a recruiting agent to the nucleus protein kinase C – zeta (PKC – zeta). Nuclear 3,4,5-IP₃ has been shown to mimic the anti-apoptotic effect of NGF (20). Protein kinase C beta – a classical PKC is located in the nucleus (21). It phosphorylates nuclear IP₃R *in vivo* and consequently enhances IP₃-mediated nuclear calcium release (22).

While the action of IP₃ and IP₃R in the nucleus has been well defined the role of inositol 1,3,4,5-tetrakisphosphate (IP₄) in the nucleus has not received the attention it deserves, although it recruits a calcium pool in the nuclear envelope (23, 24) and its receptor IP₄R, has been located on the nuclear membranes (25). High affinity nuclear IP₄R has been purified and it is a 74 kDa protein (26) distinct from various other IP₄Rs (27, 28, 29). Inositol hexakisphosphate (IP₆) is also implicated in the nuclear function (30).

Studies performed with the purified inner and outer nuclear membranes demonstrated that the nucleus is endowed with its own nuclear Ca²⁺-pump ATPase, a SERCA2b isozyme located on the outer nuclear membrane (25). The nuclear Ca²⁺-ATPase (NCA) is phosphorylated by cAMP-dependent protein kinase (PKA) leading to its enhanced calcium pumping activity (31) as well as facilitating transport of 10 kDa Calcium Green -1 dextran to the nuclear envelope.

In this review, we highlight that nuclear Calcium signaling occurs by the transport of calcium into the nuclear envelope by nuclear located specific molecules, such as Ca²⁺-ATPase and IP₄R are located on the outer nuclear membrane (25). The nuclear Ca²⁺-ATPase discharges its function depending on the external free calcium concentration and phosphorylation (31) by PKA. This establishes a cross-talk between cAMP and calcium at the level of cell nucleus. The nuclear envelope, in this scenario, serves as a transient store for the nuclear calcium. The calcium is released from the nuclear envelope into the nucleoplasm by the opening of IP₃R channel, located on the inner nuclear membrane (25), on the availability of IP₃. The action of inner membrane IP₃R is regulated through its phosphorylation by native nuclear PKC (22). According to the mechanism outlined in this review, a holistic picture has emerged where PKA, PKC, Ca²⁺-ATPase and IP₃R are key players in modulating an independent regulation of nuclear calcium homeostasis.

3. DISCUSSION

3.1. Isolated nuclei – rupturing and resealing of the nuclear membrane

In the beginning when claims for the nuclear events were advanced, particularly the nuclear location of IP₃R (1) or ryanodine receptor (32, 33), the most formidable challenge confronted was that the isolated nuclear studies were in fact free from cytoplasmic contaminants. In particular there was serious concern of microsomal contaminants of nuclei since the outer nuclear membrane, *in situ*, is continuous with the endoplasmic reticulum. We have addressed this concern in some detail (1, 25). Based on the [³²P]IP₃ binding studies and microsomal marker enzymes activity at each step of nuclei purification procedure (Table 1), we have established

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Table 2. Marker enzyme activity in the nuclear membranes

Fractions	NAD pyrophosphorylase		Mannose-6-phosphatase		NADPH cytochrome c reductase	
	Total activity $\mu\text{mol}/\text{min}$	Specific activity $\text{nmol}/\text{min}/\text{mg}$ protein	Total activity $\mu\text{mol}/\text{min}$	Specific activity $\text{nmol}/\text{min}/\text{mg}$ protein	Total activity $\mu\text{mol}/\text{min}$	Specific activity $\text{nmol}/\text{min}/\text{mg}$ protein
Liver homogenate	27.3 \pm 2.696 (100)	4.04 \pm 0.4	628 \pm 62.00 (100)	93.3 \pm 9.3	57.0 \pm 5.64 (100)	9.5 \pm 0.91
Isolated intact nuclei	7.06 \pm 0.705 (25.8)	24.3 \pm 2.4	122 \pm 12.10 (19.4)	332 \pm 33.1	0.8 \pm 0.07 (1.4)	3.2 \pm 0.30
Outer nuclear membrane	0.02 \pm 0.002 (0.074)	4.82 \pm 4.7	3.4 \pm 0.31 (0.55)	803 \pm 80.0	0.06 \pm 0.06 (0.11)	14.4 \pm 0.14
Inner nuclear membrane	1.45 \pm 0.140 (5.3)	70.0 \pm 7.0	0.414 \pm 0.04 (0.066)	20.0 \pm 1.9	0.025 \pm 0.002 (0.04)	1.2 \pm 0.12

Outer and inner nuclear membranes were separated from the nuclear envelope as described (25). NAD pyrophosphorylase activity was determined by monitoring spectrophotometrically the formation of NADH at 340 nm. Mannose-6-phosphatase activity was determined by measuring the absorbance at 730 nm. NADPH cytochrome c reductase was determined by monitoring the reduction of cytochrome c at 550 nm. NAD pyrophosphorylase activity with the inner membrane was enriched coupled with decreased NADPH cytochrome c reductase activity as compared with the intact nuclei. Similarly, mannose-6-phosphate activity was enriched with the outer membrane as compared with the nuclei. These data are mean \pm SEM of two independent preparations. Figures in parentheses represent the percentage.

without any shadow of doubt that the final nuclear preparation was devoid of microsomal association. Increased specific and total [^{32}P]IP₃ binding activity observed in the postnuclear material obtained from the nuclear preparation derived from the starting liver homogenate supplemented with exogenous microsomes, fully attested that the adequate procedure was adopted to circumvent possible microsomal association for isolation and purification of nuclei (see 1 for details).

Further support that the isolated nuclei were free from microsomal contaminants is found from the isolated inner and outer membranes integrity (25). NAD pyrophosphorylase is considered as a reliable nuclear marker (34), whereas mannose-6-phosphatase activity is common to both nucleus and microsomes (35), although at different levels. In the outer nuclear membrane preparation there occurred an enriched mannose-6-phosphatase activity as contrasted from that of the inner nuclear membrane (Table 2). The NAD pyrophosphorylase activity was enriched in the isolated inner membrane and may serve as its marker.

The electron microscopic examination of the nuclear membranes (see ref. 25) revealed that the inner nuclear membrane was vesicular with lamin lying on its inner side, antilamin antibody identifying lamin was only accessible upon low salt treatment, confirming that the inner membranes, as isolated, were in right-side-in orientation. The outer nuclear membrane was represented by a membranous sheath like structure. A successful separation of inner and outer nuclear membranes (25) from the isolated nuclei has established that the two nuclear membranes are, not only structurally different from one and another but also differ in their calcium signaling molecular components, confirming that the nucleus has its own machinery for inositol lipids-mediated biological functions (13, 16, 36).

In another instance, nuclear and cytoplasmic extractions were carried out (18). A comparison (Table 3) of NAD pyrophosphorylase, mannose-6-phosphatase and NADPH cytochrome c reductase activity confirmed that the

nuclear preparation was free, as best as experimentally feasible, from the cytoplasmic constituents. Once again NAD pyrophosphorylase was confirmed to be a unique nuclear marker (Table 3). Data documented here (Table 1 – 3) provided full justification that isolated nuclei are a dynamic organelle, free from microsomal contaminants, mimicking some of the physiological functions, as *in situ*, situation. A number of laboratories have successfully isolated nuclei from both tissues and cells in culture (2, 12, 14, 16, 32, 33, 37, 40).

The vesicular configuration with right-side-in orientation (25) of the isolated inner nuclear membrane indicated the rupture of the continuity of the outer nuclear membrane with ER during the isolation procedure and the resealing of the ruptured outer nuclear membrane. This has been observed by many investigators (1, 2, 32, 33, 38, 39).

3.2. Nuclear inositol 1,4,5-trisphosphate receptor (IP₃R)

The specificity of IP₃R located on the nuclear membrane was verified by competitive displacement of [^{32}P]IP₃ (Figure 1) by various inositol phosphates. The IP₃ specific binding site was further characterized by their rank order of the ligands studied i.e., IP₃ > 3,4,5-IP₃ > IP₄ > ATP. The binding was sensitive to the pH of the medium and was enhanced when pH was raised from 7.5 to 8.5 (1). IP₃ and heparin were the most potent inhibitors of this binding. The total binding was ~ 20 % of [^{32}P]IP₃ added in the binding assay medium.

What is the role of nuclear IP₃ binding site in the nuclear calcium movement was the most appropriate question to address. Nuclei were loaded with $^{45}\text{Ca}^{2+}$ in the presence of ATP. In the absence of ATP no $^{45}\text{Ca}^{2+}$ could be loaded to the nuclei (Figure 2) Introduction of 10 μM IP₃ in the medium bathing the $^{45}\text{Ca}^{2+}$ loaded nuclei caused a robust release of nearly half of the $^{45}\text{Ca}^{2+}$ loaded in a time dependent manner. The calcium released by IP₃ was rapid and transient with the maximum ~1 min after the IP₃ addition (Figure 2).

The continued presence of ATP indicated the occurrence of $^{45}\text{Ca}^{2+}$ reuptake after initial transient release

Table 3. Marker Enzyme Activity in Nuclear and Cytoplasmic Extracts

Fraction	NAD pyrophosphorylase		Mannose-6-phosphatase		NADPH cytochrome c reductase	
	Total activity	Specific activity	Total activity	Specific activity	Total activity	Specific activity
liver homogenate	20.5 ± 2.0	3.50 ± 0.35	435.00 ± 43.4	78.70 ± 7.85	52.50 ± 5.20	14.50 ± 1.4
nuclear extract	5.33 ± 0.5	17.80 ± 1.77	85.60 ± 8.53	156.80 ± 15.66	2.55 ± 0.25	5.80 ± 0.5
cytoplasmic extract	1.06 ± 0.1	4.20 ± 0.40	32.10 ± 3.20	94.00 ± 9.38	46.40 ± 4.62	18.00 ± 1.7

NAD pyrophosphorylase activity, mannose-6-phosphatase activity and NADPH cytochrome c reductase activity were determined as described in the Table 2. The total activity is expressed as micromoles per minute, and the specific activity is expressed as the nanomoles per minute per milligram of protein. These data are mean ± SEM of two independent preparations.

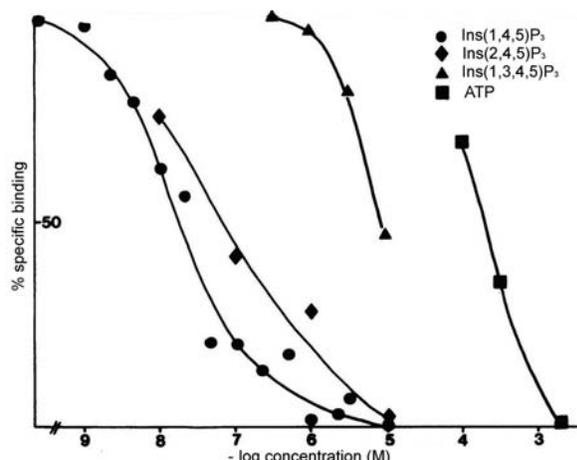


Figure 1. Characterisation of specific $[^{32}\text{P}]\text{IP}_3$ binding to isolated rat liver nuclei. $[^{32}\text{P}]\text{IP}_3$ binding was done in an Eppendorf tube at 0°C (on ice) for 30 min in a medium containing 50 mM Tris-HCl (pH 8.0) and 2 mM EDTA. The protein was 1 mg/ml and the total volume of the assay mixture was 400 μl . Nonspecific binding was determined in the presence of nonradioactive IP_3 . These experiments were performed twice in quadruplicate with replicates varying by < 10 %.

(Figure 2). This was circumvented when an ATP-dissipating system (hexokinase and glucose) was introduced in the medium.

3.3. IP_3R located on the Inner nuclear membrane

The specificity of nuclear IP_3R was further investigated on the inner nuclear membrane (25) analyzing competitive displacement of $[^3\text{H}]\text{IP}_3$ by various inositol phosphates (Figure 3A). IP_3 was found the most potent inhibitor of binding. Binding was also inhibited, although to a lesser extent, by the 2,4,5- IP_3 , 3,4,5,6- IP_4 , and IP_5 (Figure 3A). Heparin at 10 $\mu\text{g}/\text{ml}$ completely abolished $[^3\text{H}]\text{IP}_3$ binding. No meaningful $[^3\text{H}]\text{IP}_3$ binding activity was detected with the outer nuclear membrane preparation. The functional efficiency of the IP_3R located to the inner nuclear membrane was further verified by comparing the K_d and B_{max} values obtained from the inner nuclear membrane (Figure 3B) and the intact nuclei (Figure 3C). Scatchard plots of saturation isotherms revealed a single class of binding sites with $K_d = 5.5$ nM and $B_{\text{max}} = 1205$ fmol/mg protein with the inner nuclear membrane. Similar Scatchard analysis with the intact nuclei gave $K_d = 7.0$ nM and $B_{\text{max}} = 385$ fmol/mg protein. Increased B_{max} values in the case of inner nuclear membrane indicate a rise in receptor density upon membrane purification. Almost no

change in K_d values in the two nuclear preparations attests to an identical functional receptor in the intact nucleus and the inner membrane derived from it, affirming that the inner membrane is the site of location of nuclear IP_3R .

3.4. Nuclear PKC phosphorylates IP_3R *in vivo*

The inner membrane location of nuclear IP_3R is further confirmed by the Western blotting with anti- IP_3R antibody (Figure 4). Anti- IP_3R antibodies raised against purified rat cerebellum IP_3R revealed on Western blotting rat cerebellar extract or rat microsomal fraction, a distinct classical 260 kDa protein band (Figure 4A). In the nuclear extract the same anti- IP_3R antibody immunoblotted a 220 kDa protein band (Figure 4A). Likewise, the same 220 kDa protein band was immunoblotted in the inner nuclear membrane (Figure 4B). No such immunoreactive protein was revealed with the outer nuclear membrane. A distinct 220 kDa protein band immunoblotted only with the inner nuclear membrane and not with the outer nuclear membrane affirms the binding data that indeed IP_3R is located on the inner nuclear membrane.

That the nuclear IP_3R is a 220 kDa protein distinct from its counterpart – a 260 kDa protein located in the rat cerebellum or rat microsomes (Figure 4A) - found further support when an almost *in situ* situation, nuclear IP_3R represented by 220 kDa protein (Figure 4C) was phosphorylated with native nuclear PKC (21, 41). The phosphorylation of IP_3R by nuclear located endogenous PKC enhanced the capacity of IP_3 (Figure 5) to release calcium (22).

The identification of the nucleoplasmic reticulum structure (42) and location of IP_3R to this structure in SKHep1 cells confirmed that the nucleus is endowed with a distinct site for the storage and release of Ca^{2+} .

3.5. Mechanism of nuclear IP_3 generation

It has been well established, over the years, from our own work (1, 22, 23, 25) and the work of many investigators (32, 33, 36, 38, 43), that it is the nuclear IP_3 and not the cytosolic IP_3 that regulates nuclear calcium signaling. But the mechanism by which nuclear PLC is stimulated to generate IP_3 within the nucleus was missing. We have fulfilled this discrepancy by demonstrating that nuclear PLC-gamma 1 is activated by EGF nuclear signaling *in vivo* (18) and thus documented for the first time a detailed mechanism for nuclear IP_3 generation. It has not been possible to determine the level of IP_3 in the nucleus so far, presumably due to the lack of suitable techniques to capture nuclear IP_3 (discussed further below).

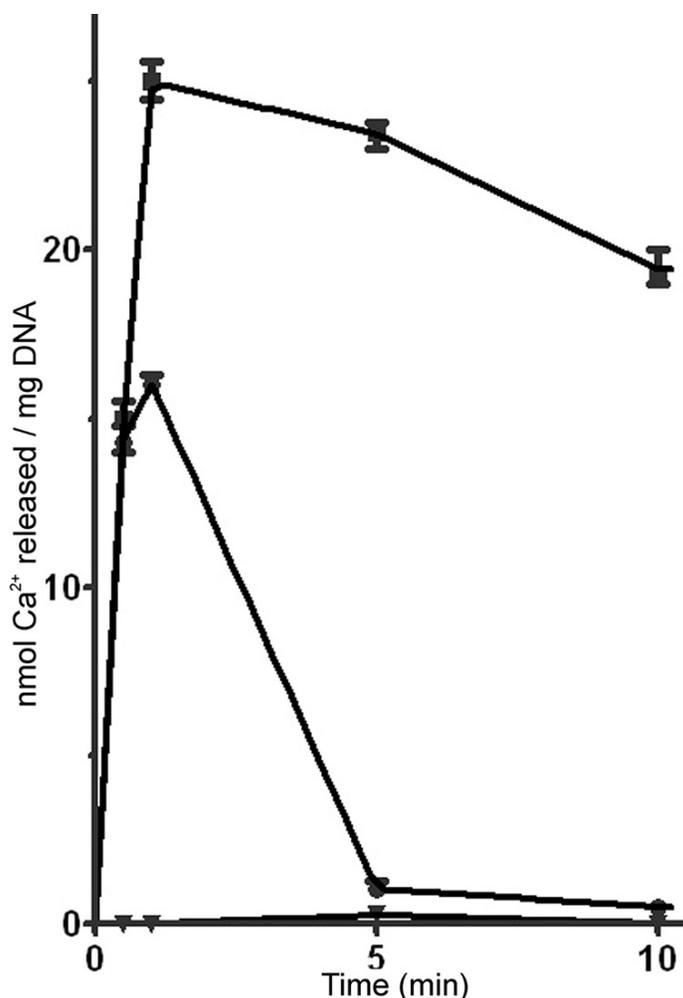


Figure 2. IP₃-induced ⁴⁵Ca²⁺ release from nuclei. Isolated nuclei (0.1 mg of DNA per tube) were loaded with ⁴⁵Ca²⁺ by incubating for 1 hr at 37°C in the presence of 1 mM ATP and traces of ⁴⁵Ca²⁺ (2 μCi/ml). The uptake was stopped by placing the nuclei on ice. After a 15-min incubation on ice, the nuclei were preincubated (2 min) at 37°C and the release was initiated by addition of 50 μl of IP₃ (final concentration, 10 μM). ⁴⁵Ca²⁺ release, after a given time, was defined as the radioactivity loaded (time 0) minus the radioactivity trapped on the filter. These experiments were carried out on three independent nuclear preparations in quadruplicate with replicates varying by <10%. ▼, 50 μl of buffer with no IP₃; ●, IP₃ in the presence of ATP; ■, IP₃ plus ATP plus glucose plus hexokinase.

The injection of EGF to mouse revealed upon immunoblotting with isozyme specific anti-PLC-gamma 1 antibody, two protein bands in the liver nuclear extract, one at 150 kDa and the other at 120 kDa (Figure 6A). When immunoblotted with the anti-phosphotyrosine antibody, the EGF treatment at 10 or 30 min tyrosine phosphorylated the 120 kDa protein and not the 150 kDa (Figure 6B). The 120 kDa protein was immunoprecipitated (18) with anti-PLC-gamma 1 antibody from the nuclear extract of control and EGF treated mice, followed by immunoblotting with anti-phosphotyrosine antibody, further affirming that at 10 or 30 min, EGF treatment phosphorylated (Figure 6C) the 120 kDa protein at the tyrosine (44). Furthermore, the nuclear 120 kDa protein was immunoprecipitated with isozyme-specific PLC-gamma 1 antibody and was sensitive to a specific blocking peptide (SFEANQQPFEDFRI) indicating that the 120 kDa protein (Figure 6D) band, which is a

target of EGF-mediated signaling in the nucleus, is a PLC-gamma 1 related protein.

Likewise, the cytoplasmic extract obtained from control and EGF-treated mice when immunoblotted (18) with anti-PLC-gamma 1 antibody revealed a single 150 kDa protein band (Figure 6E) which was tyrosine-phosphorylated as observed with anti-phosphotyrosine antibody (Figure 6F). This indicated that the occurrence of 120 kDa protein in response to EGF-mediated signaling to the nucleus is an exclusively nuclear specific event.

To further probe the function of the 120 kDa protein fragment that we considered a proteolysis product of native nuclear 150 kDa PLC-gamma 1 (18), the role of nuclear membrane (45) located EGFR (19, 46) was studied. Immunoblotting the mouse liver nuclear extract with anti-EGFR antibody revealed a 170 kDa protein band (Figure

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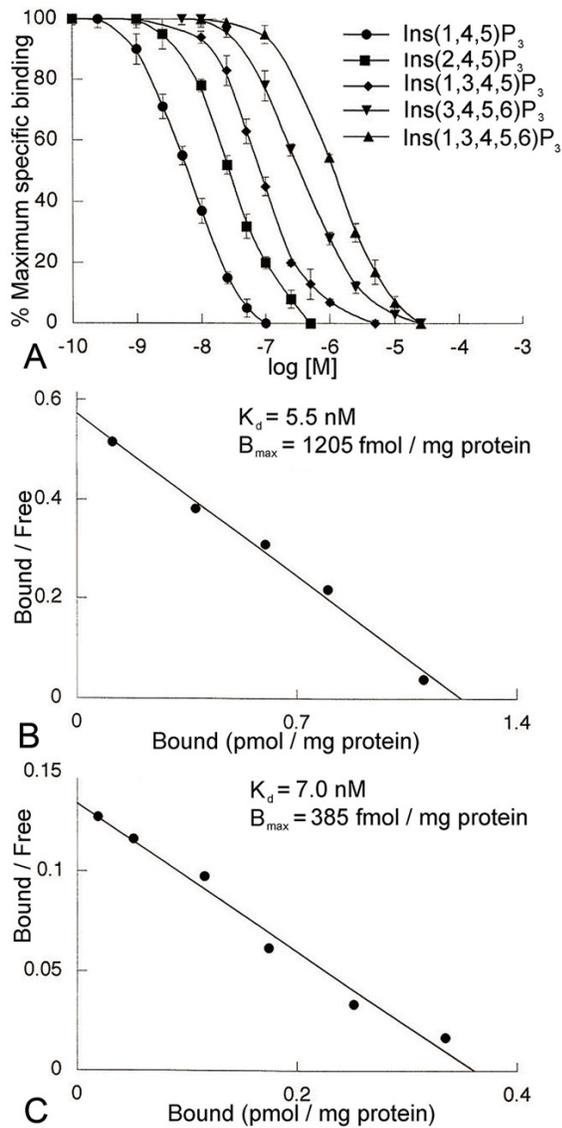


Figure 3. $[^3\text{H}]\text{IP}_3$ binding to purified inner nuclear membrane. (A) $[^3\text{H}]\text{IP}_3$ binding with various competing ligand. $[^3\text{H}]\text{IP}_3$ binding was carried out in an Eppendorf tube at 0°C (on ice) for 10 min in a medium containing 50 mM Tris-HCl, pH 8.0, and 2 mM EDTA. The inner membrane protein was 0.1 mg, and the total volume of assay medium was 400 μl . Each assay was carried out in quadruplicate. Numbers in parentheses on the panel denote the respective isomer of various inositol phosphate derivatives. Scatchard plots of $[^3\text{H}]\text{IP}_3$ binding to the inner nuclear membrane (B) and to the intact nuclei (C). The K_d and B_{max} were determined by Scatchard analysis under classical binding conditions, i.e. by using progressively increasing concentrations of $[^3\text{H}]\text{IP}_3$. These experiments were performed on two independent nuclear preparations in quadruplicate, with replicates varying by $<10\%$.

7A). The anti-EGFR antibody immunoprecipitates derived from the nuclear extract of the control and EGF-treated mice, and immunoblotted with anti-active EGFR antibody,

confirmed that in response to a 10 or 30 min EGF treatment, EGFR was phosphorylated on tyrosine (Figure 7B). Likewise, immunoprecipitation either with anti-active EGFR (Tyr¹¹⁷³) or with anti-active EGFR (Tyr¹⁰⁶⁸) antibodies, immunoblotted with the respective antibodies (Figure 7C and 7D), confirmed that at the 10 or 30 min EGF injection, nuclear membrane EGFR was phosphorylated at the tyrosine (44) and hence activated.

Furthermore, the time course of 120 kDa protein tyrosine phosphorylation was coincident with tyrosine phosphorylation and consequent to an activation of nuclear membrane located EGFR, suggesting that EGF nuclear signaling activates nuclear EGFR that in turn phosphorylates 120 kDa protein (18). In support of this hypothesis, the association between activated EGFR and 120 kDa protein phosphorylated at the tyrosine was revealed when immunoprecipitates obtained with a number of antibodies from the nuclear extract of the EGF-treated mice was immunoblotted with anti-PLC-gamma 1 antibody, a 120 kDa protein band was detected in each case (Figure 7E to 7G). Confirmation of the association between 120 kDa protein and EGFR and hence the mechanism of activation of nuclear PLC-gamma 1 was once again reinforced, as revealed by the detection of 170 kDa EGFR in the immunoprecipitates of anti-PLC-gamma 1 antibody, under identical conditions and within the same time frame (Figure 7H).

The immunoprecipitated 120 kDa protein shows PI hydrolytic activity (Figure 8). We attribute this activity to the 120 kDa protein since it is this moiety and not the native 150 kDa moiety which is tyrosine phosphorylated in the nucleus in response to the EGF signaling. Second, under our experimental paradigm no other contaminating protein was detected on the gel. However, this does not rule out absolutely that the immunoprecipitated 120 kDa moiety may not be associated with minor undetected proteins. Notwithstanding, it is unlikely that the PI hydrolysis activity that we attribute to the 120 kDa protein is contributed by some undetectable moiety.

Thus, there are ample reasons, verified and tested experimentally, to propose that the 120 kDa protein band that we have observed in the mouse liver nuclei is, in fact, a protein fragment of the nuclear PLC-gamma 1. It is known that activated PLC-gamma 1 binds PIKE - a nuclear GTPase (12) - consequently activating nuclear PI(3)K. This precept was put to verification in our experimental paradigm. The immunoprecipitates obtained with anti-PIKE antibody immunoblotted with anti-PLC-gamma 1 antibody revealed the 120 kDa protein band at 10 or 30 min of EGF treatment (Figure 9A). Conversely, immunoprecipitate with anti-PLC-gamma 1 antibody and immunoblotted with anti-PIKE antibody revealed a 98 kDa PIKE band (Figure 9B) association with PLC-gamma 1, upon later activation. Interaction between PI(3)K and the 120 kDa protein band was examined by immunoprecipitating this enzyme with the anti-p110 alpha antibody, in the control and EGF-treated mice nuclear extract and immunoblotting with anti-PLC-gamma 1 antibody (Figure 9C). This interaction was reinforced by

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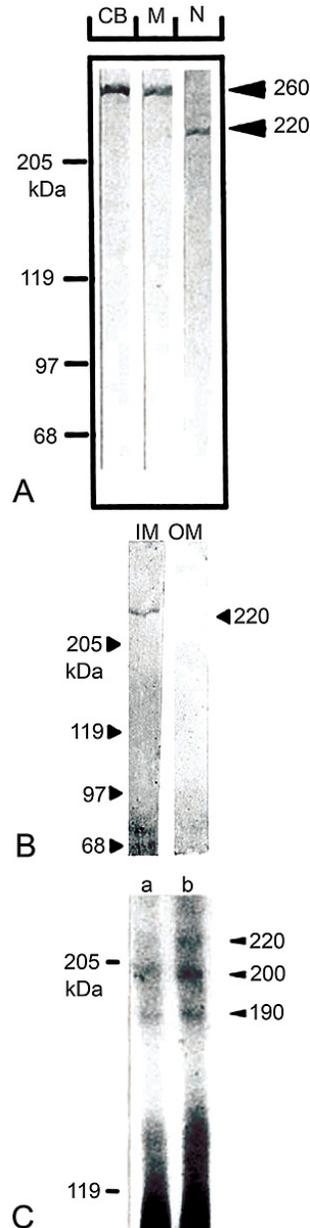


Figure 4. Identification and PKC phosphorylation of nuclear IP₃R. (A) Rat liver nuclear (N), microsomal (M), and cerebellar (CB) extracts were separated on SDS-PAGE using 7.5% polyacrylamide. Subsequent to electrophoresis, they were transferred to nitrocellulose membranes. Membranes were incubated with anti-IP₃R sera at a dilution of 1:200 (anti-rabbit) or 1:500 (anti-goat) for 1 hour at room temperature. The immunocomplex was revealed by alkaline phosphatase Misty purple reagent. 100 µg of cerebellar, microsomal, or nuclear protein was loaded on each gel. On the left-hand side is the molecular mass in kilodaltons of marker proteins. Microsomal and cerebellar preparations were used as positive control showing 260 kDa immunoreactive proteins in these preparations. Whereas the same antibodies recognized a 220 kDa protein in rat liver nuclei, a few minor proteins seen are due to nonspecific interaction of antibodies. (B) Inner membrane (IM) and outer nuclear membrane (OM) were electrophoresed on 8% acrylamide and transferred to nitrocellulose membrane. 10 µg of membrane protein was loaded on each lane. Note that the 220 kDa protein band identified with the inner nuclear membrane is the nuclear IP₃R, which was phosphorylated by protein kinase C *in vivo* conditions. (C) Phosphorylation of nuclear IP₃R by PKC. Nuclei were treated with TPA (b), and, in parallel, a control was run where nuclei were not treated with TPA (a). Subsequent to TPA treatment (or control), nuclei were centrifuged. The pellets were resuspended. Radiolabeling with [³²P]ATP. IP₃R was immunoprecipitated with anti-IP₃R sera at a dilution of 1:100. On the left-hand side is the migration of myosin as a molecular marker (205 kDa). 30 µg of protein was loaded on each lane.

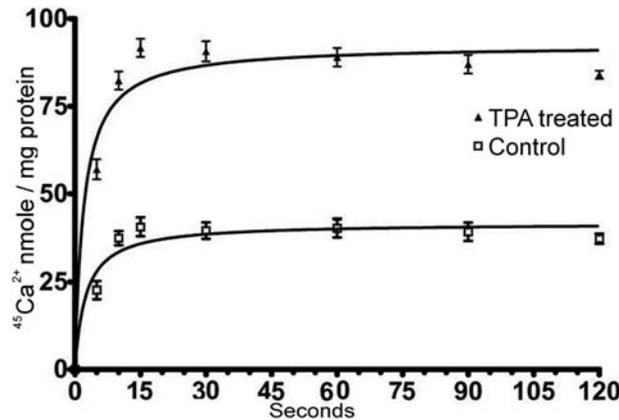


Figure 5. IP₃ mediated ⁴⁵Ca²⁺ movement upon TPA treatment of nuclei. Nuclei TPA-treated and control were loaded separately with calcium by incubating with traces of ⁴⁵Ca²⁺ (2 μCi/ml) in the presence of 1 mM ATP for 1 h at 37°C. The loading was terminated by placing the nuclei on ice for 15 min. Nuclei were preincubated at 37°C, and the ⁴⁵Ca²⁺ release was initiated by adding 10 μl IP₃ (10 μM final concentration). ⁴⁵Ca²⁺ release, after the indicated time, was defined as the radioactivity loaded (time 0) minus the radioactivity trapped on the filter. These experiments were carried out on three different nuclear preparations in quadruplicate.

observing stimulated nuclear PI(3)K activity (Figure 10) in the anti-p110 alpha antibody immunoprecipitate obtained from the nuclear extract of control and EGF-treated mice (Figure 10).

Stimulated PLC-gamma 1 (44) targets PIP₂, generating IP₃ and DAG. In the liver nuclear extract, a 3-fold rise in the nuclear DAG concentration (Figure 11) after EGF treatment for 10 or 30 min, corresponds with the 120 kDa protein moiety tyrosine phosphorylation and, consequently, its stimulation in the nucleus (discussed further below).

Concrete experimental evidence is placed here to support the hypothesis that the 120 kDa protein fragment, which is a target for nuclear activated EGFR (44) and a sequel to EGF nuclear signaling (40), binds PIKE and PI(3)K and triggers nuclear PI(3)K activation. Hence, it was considered legitimate to obtain the partial N-terminal and C-terminal sequence analysis (18) of the 120 kDa protein fragment. This sequence analysis revealed that the 120 kDa protein fragment is PLC-gamma 1 without N-terminal amino acid residues 1 - 243 (when compared with the rat PLC-gamma 1 sequence). The 10-partial sequence analysis revealed that the 120 kDa protein contains the PELCQUSSE sequence at its N-terminal end at the RTRVNGNRL sequence at its C-terminal end (18). With the starting N-terminal amino acid identified as Pro²⁴⁴, in the 120 kDa protein, it may be safely proposed that the site of cleavage, in nuclear PLC-gamma 1, is located between Arg²⁴³ and Pro²⁴⁴.

Several data obtained in this study support the fact that the 120 kDa protein is derived from the native nuclear 150 kDa PLC-gamma 1. First, the 120 kDa protein was immunoprecipitated with isozyme-specific anti-PLC-gamma 1 antibody. Second, the 120 kDa protein, like the 150 kDa PLC-gamma 1, was sensitive to the isozyme-

specific blocking peptide. Third, the 10 partially sequenced amino-acids at the N-terminal end are identical to those of the N-terminal sequence Pro²⁴⁴-Glu²⁵³ found in rat PLC-gamma 1 (47). Fourth, the C-terminal amino acid partial sequence was identical to the PLC-gamma 1 C-terminal end. Fifth, it is known that PLC-gamma 1 interacts with PIKE through its SH3 domain (12) spanning from amino acid residues 797 to 841 (48), which is intact in the 120 kDa protein fragment. Sixth, more than a 3-fold increase in nuclear DAG levels (Figure 11) at 10 or 30 min of EGF treatment, albeit there are other sources of DAG generation (49).

Since the techniques to determine nuclear IP₃ remain obscure and it has not been possible to quantify nuclear generated IP₃, it is reasonable to speculate that nuclear-generated IP₃ stays long enough to mobilize calcium from the nuclear envelope to the nucleoplasm, but not long enough to be determined with the current tools. In this context, an analogy can be derived from the ryanodine receptors (RyR) that are also located on the inner nuclear membrane (32, 50) with their agonist binding sites facing toward the nucleoplasm. Functionally active CD38/ADP ribosyl cyclase (50) is located on the inner nuclear membrane, generating cADP ribose which is an agonist for RyR (like IP₃ for IP₃R). This analogy between cADP ribose and IP₃ makes it most likely that IP₃ is generated in the nucleus and binds to the IP₃R located to the inner nuclear membrane, facilitating calcium movement from NE to the nucleoplasm.

Partial proteolysis of PLC-gamma 1 in the nucleus, according to the data documented here, appears to be a mechanism by which nuclear PLC-gamma 1 is stimulated. Future research will be needed to understand the mechanism of nuclear PLC-gamma 1 proteolysis. The field (51, 52) of partial proteolysis has begun to unravel the understanding of signal transduction pathways. For

Inositol and calcium signals in the nucleus

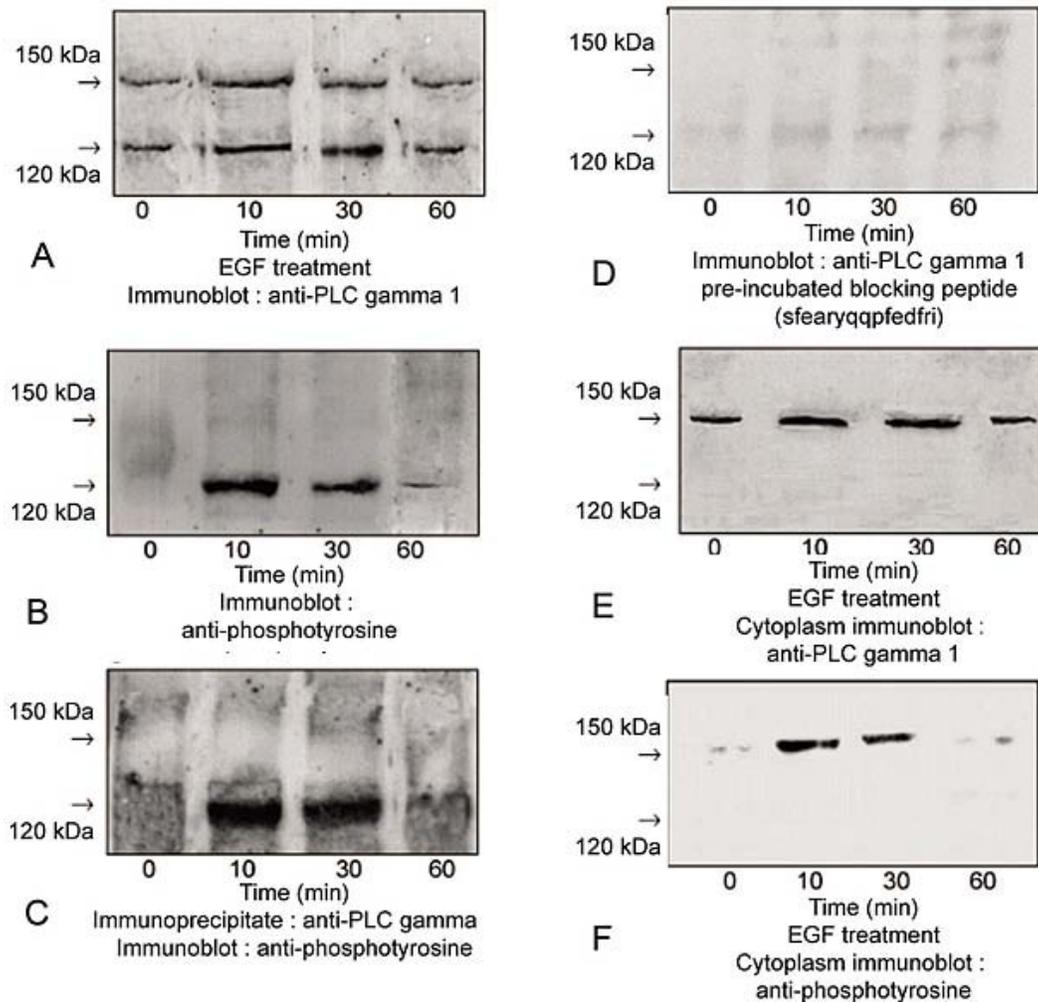


Figure 6. Immunoblot with anti-PLC-gamma 1 antibodies. (A) Mouse liver nuclear extracts (60 μ g of protein), obtained at the indicated time after EGF had been injected intraperitoneally, were immunoblotted with isozyme specific anti-PLC-gamma 1 antibody, depicting two protein bands 150 and 120 kDa. (B) Western blotting with anti-phosphotyrosine antibody (as in panel A) revealed a tyrosine-phosphorylated 120 kDa band at 10 and at 30 min after EGF injection. (C) Immunoprecipitate obtained with anti-PLC-gamma 1 antibody from nuclear extract (300 μ g of protein) immunoblotted with anti-phosphotyrosine antibody confirming that the 120 kDa protein band was tyrosine-phosphorylated after EGF treatment for 10 or 30 min. (D) Immunoblot as in panel A, except that the anti-PLC-gamma 1 antibody was preincubated with a specific blocking peptide (SFEANQQPFEDFRI), affirming that the two protein bands were related to PLC-gamma 1. (E) Mouse liver cytoplasmic extracts (70 μ g of protein) obtained as in panel A were immunoblotted with anti-PLC-gamma 1 antibody showing a single 150 kDa protein band. (F) Western blotting with anti-phosphotyrosine antibody (as in panel E) revealed a tyrosine-phosphorylated cytoplasmic 150 kDa PLC-gamma 1 in response to EGF treatment. Amount of protein loaded on each lane were: panels A & B, 60 μ g; panels C & D, 300 μ g; panels E & F, 70 μ g.

instance, proteolysis of suprachiasmatic nucleus circadian oscillatory protein (SCOP) contributes to activation of MAP Kinase (53).

3.6. Nuclear Ca^{2+} -ATPase (NCA) - location and characterization

A single immunoreactive 105 kDa protein band (Figure 12) was identified with the outer nuclear membrane (25) employing anti- Ca^{2+} -ATPase antisera raised against ER Ca^{2+} -ATPase. No such immunoreactive

protein was observed with the inner nuclear membrane, indicating that the NCA is located on the outer nuclear membrane and triggers the ATP-mediated calcium transport to the nuclear envelope (1, 32).

The NCA was immunoprecipitated after db-cAMP or forskolin treatment of HEK 293 cells in culture (37) with polyclonal goat anti-SRERCA (c-20) antibody (Figure 13A). Immunoblotting the immunoprecipitated 105 kDa protein band from the isolated nuclei with anti-

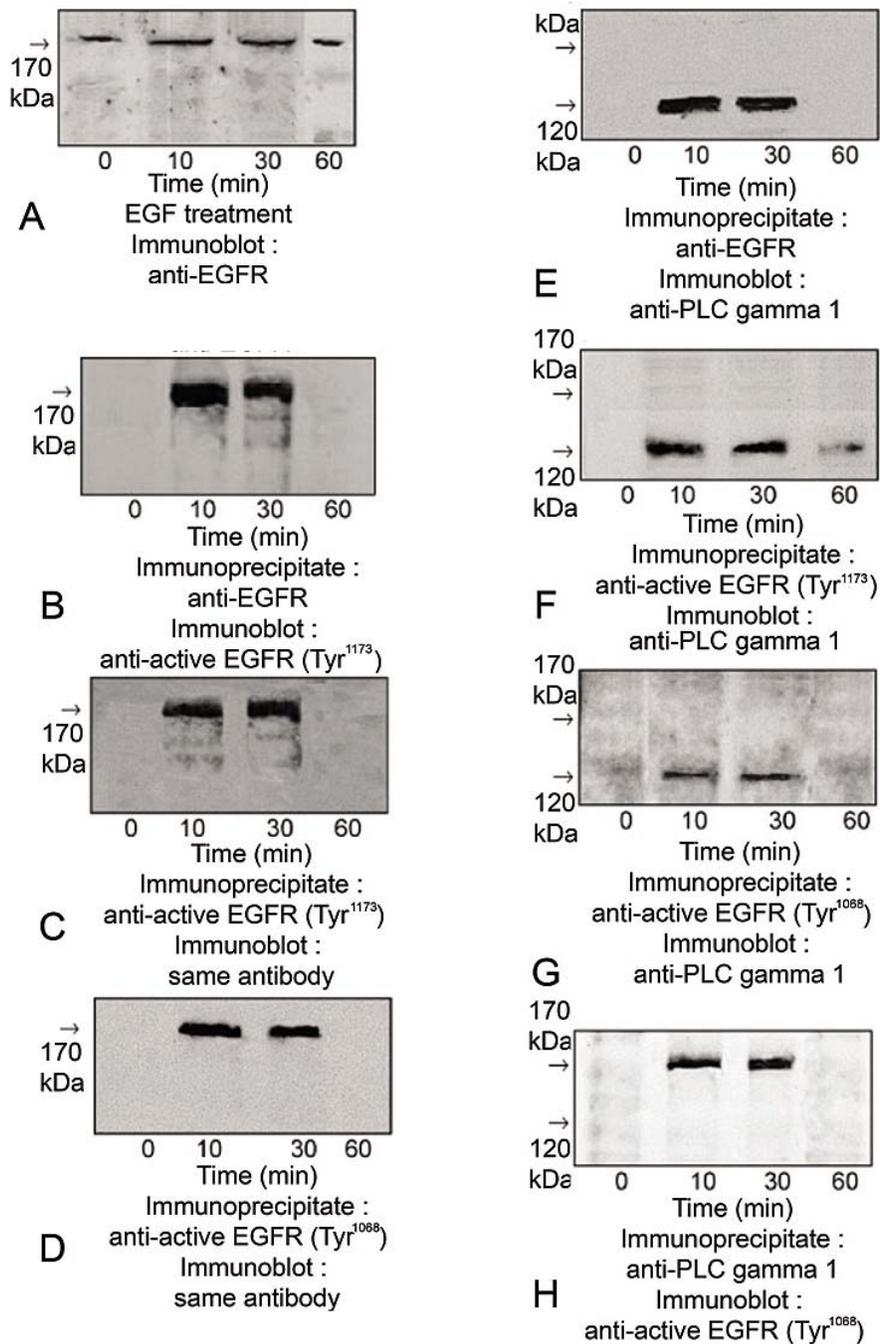


Figure 7. EGF-induced activation of nuclear membrane EGFR. (A) Nuclear extract immunoblotted (as in the legend of Figure 6) with anti-EGFR antibodies. (B) Immunoprecipitate obtained with anti-EGFR antibody from nuclear extract (300 μ g of protein) immunoblotted with anti-EGFR phosphospecific (Tyr¹¹⁷³) antibody depicting the time course of nuclear membrane EGFR tyrosine phosphorylation. (C) Immunoprecipitate with anti-EGFR phosphospecific (Tyr¹¹⁷³) antibody immunoblotted with the same antibody confirming EGFR tyrosine phosphorylation as in panel B. (D) Same as panel C with anti-EGFR phosphospecific (Tyr¹⁰⁶⁸) antibody. (E) Same as panel B immunoblotted with anti-PLC-gamma 1 antibody. (F) Same as panel C immunoblotted with anti-PLC-gamma 1 antibody. (G) Same as panel D immunoblotted with anti-PLC-gamma 1 antibody. (H) Immunoprecipitate with anti-PLC-gamma 1 antibody immunoblotted with anti-EGFR phosphospecific (Tyr¹⁰⁶⁸) antibody. In each lane 300 μ g of protein of nuclear extract were immunoprecipitated and loaded.

Inositol and calcium signals in the nucleus

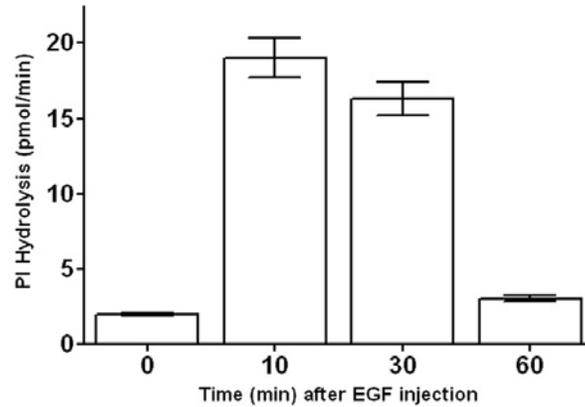


Figure 8. Phosphatidylinositol hydrolysis of 120 kDa protein. The [^3H]PI hydrolysis activity was determined in a 200 μl assay mixture containing 200 μM PI (20,000 cpm [^3H]PI). The reaction was incubated at 37°C for 15 min and the reaction was terminated by adding 1 ml of a chloroform/methanol/HCl mixture. Radioactivity in the aqueous phase was determined by liquid scintillation spectrometry.

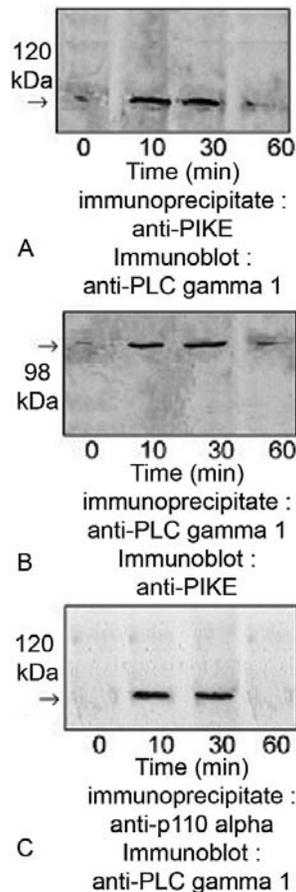


Figure 9. EGF mediated association between the 120 kDa protein and PIKE. (A) The immunoprecipitate obtained with anti-PIKE antibody from the nuclear extract (300 μg of protein) of control and EGF treated mice immunoblotted with anti-PLC-gamma 1 antibody revealed the presence of a 120 kDa protein band after EGF treatment for 10 or 30 min. No such association was seen with or without EGF treatment for 60 min. (B) Same as panel A whereby immunoprecipitate with anti-PLC-gamma 1 antibody immunoblotted with anti-PIKE antibody revealing the 98 kDa PIKE band. (C) Immunoprecipitate obtained with anti-p110 alpha antibody immunoblotted with anti-PLC-gamma 1 antibody. In each lane 300 μg of protein of nuclear extract were immunoprecipitated and loaded.

Inositol and calcium signals in the nucleus

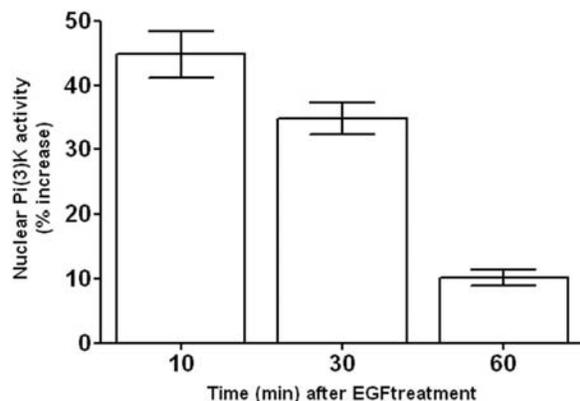


Figure 10. PI(3)K activity. Immunoprecipitate obtained with anti-p110 alpha antibody from the nuclear extract (300 μ g of protein) of EGF-treated mice depicting the percentage increase in the PI[3]K activity determined with PI and [γ - 32 P]ATP (10 μ Ci/sample). Phosphate incorporation was carried out for 15 min and the reaction was stopped. The spots resolved on oxalate-coated thin-layer chromatography plates were quantified, after autoradiography, by liquid scintillation spectrometry.

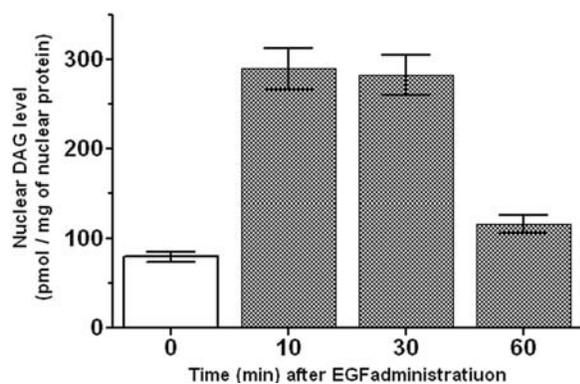


Figure 11. Nuclear diacylglycerol determination after EGF treatment. Amersham reagent system based on a radioenzymatic assay converting DAG to [32 P] phosphatidic acid in the presence of [γ - 32 P] ATP (71). [32 P] phosphatidic acid was separated on Amprep minicolumns and quantified using liquid scintillation spectrometry.

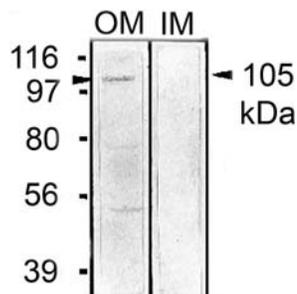


Figure 12. Western blot of NCA. Isolated nuclear membranes, inner (IM) and outer (OM), were separated on the SDS-PAGE with 8 % polyacrylamide. Antiserum raised against endoplasmic reticulum Ca^{2+} -ATPase was used. 10 μ g of protein was loaded on each lane. Note 105 kDa NCA only in the IM.

phosphoserine antibody (Figure 13B) revealed that the 105 kDa was phosphorylated with PKA (37).

To further characterize the 105 kDa protein, the isolated (54) rat liver nuclei were incubated with [γ - 32 P]ATP under various phosphorylating conditions with and without PKA (31) in the medium (Figure 14A). In the presence of 10 μ M Ca^{2+} , the 105 kDa protein was phosphorylated, but no such phosphorylation was seen at a low Ca^{2+} concentration (0.1 μ M). This indicated that the 105 kDa protein in the absence of PKA corresponds to a Ca^{2+} -dependent phosphoenzyme intermediate (55) which could be abolished after pretreatment with thapsigargin. This mode of phosphorylation was insensitive to the calmodulin inhibitor trifluoperazine, confirming that it is not due to activation of endogenous nuclear calmodulin-dependent protein kinase by Ca^{2+} . The incubation of isolated nuclei with PKA in the presence of [γ - 32 P]ATP resulted in a rapid appearance of a phosphorylated 105 kDa protein band (Figure 11A), the phosphorylation was sensitive to thapsigargin, confirming that it corresponds to a SERCA-type enzyme (56, 57).

To address the question that the 105 kDa anti-SERCA antibody-reactive and PKA-phosphorylated bands are identical, partial purification of NCA was carried out using a reactive red-120 agarose affinity chromatography. This provided reasonably enriched NCA preparations. The major protein band eluted with peak I fraction (Figure 14B) was the 105 kDa moiety recognized by anti-SERCA antiserum. Complete elution of the enzyme by using nucleotides (AMP-PNP) alone was not possible, and the remaining enzyme attached to the dye matrix was eluted with 2 M NaCl giving a second peak.

Determination of Ca^{2+} -ATPase activity confirmed that this single-step partial purification procedure yielded a significantly enriched and active enzyme preparation (Table 4). By itself detergent solubilization of nuclei with C_{12}E_9 increased specific activity, presumably because of removal of insoluble material by centrifugation. The most significant enrichment in specific activity was observed with peak I fractions (Table 4), with an increase of more than 150-fold as compared with that of isolated nuclear preparation.

Phosphorylation by PKA of concentrated peak I and II samples in the presence of [γ - 32 P]ATP followed by SDS-PAGE, immunoblotting with anti-SERCA antibody and autoradiography of the same blots showed that the antibody used immunoblots with the ^{32}P -labeled 105 kDa protein band. This finding confirmed that NCA is a PKA substrate (Figure 14).

PKA-mediated phosphorylation and subsequent stimulation of calcium pumping activity into heart mitochondria was reported (58).

The stoichiometry of phosphorylation of NCA by PKA was 0.28 mol of phosphate incorporated/mol NCA when nonpurified intact nuclei were used and 0.76 mol of phosphate incorporated/mol for partially purified peak I

Inositol and calcium signals in the nucleus

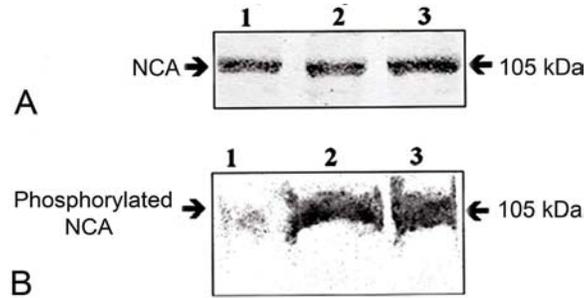


Figure 13. NCA phosphorylation in the nucleus of HEK293 cells by PKA. Nuclear extract obtained from cultured HEK293 cells db-cAMP of forskolin treatment was separated on 10 % SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted (A) with anti-SERCA antibody, (B) depicts the immunoblot revealed with anti-phosphoserine antibody. 300 μ g protein of nuclear extract was immunoprecipitated with anti-SERCA antibody. The immune complex was developed using the ECL system.

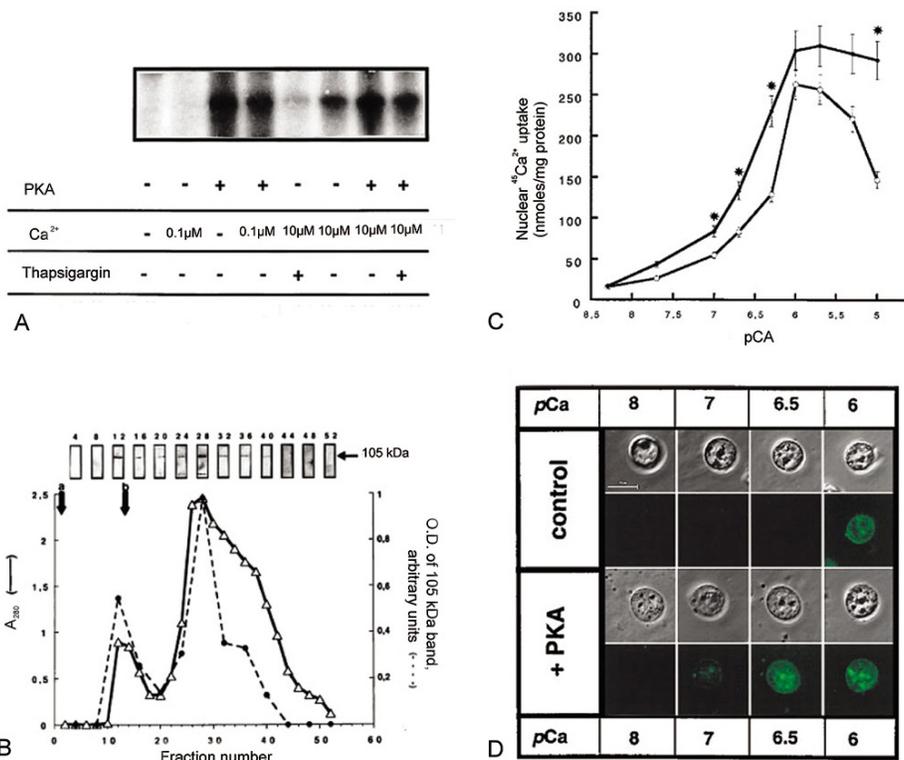


Figure 14. Characterization of NCA : phosphorylation, partial purification and nuclear transport. (A) Nuclei (16 μ g) were incubated with 20 Units of PKA catalytic units in a 50 μ l of the medium with 0.1 μ M okadaic acid and 5 mM NaF. (B) Reactive red-120 agarose column affinity chromatography was performed as described (31). NCA elution was monitored by immunoblot analysis of aliquots taken from every fourth eluted fraction by using antibodies specific for SERCA2b. A 105 kDa band was observed, which was analyzed by densitometric scanning of the immunoblots (broken line). Two bands were detected in certain fractions corresponding to peak II. Elution of proteins was measured by absorbance at 280 nm (A_{280} , solid line). (C) Isolated nuclei before (\circ) and after phosphorylation (\bullet) were incubated at 37°C for 5 min in the presence of 1 mM ATP. Calcium chloride was added into the medium bathing nuclei so as to give the indicated free calcium concentration. Traces of $^{45}\text{Ca}^{2+}$ were also present in the medium (2 μ Ci/ml; 1 Ci = 37 Gbq). Ca^{2+} uptake was terminated by filtering under vacuum over GF/B Whatman glass fiber filters, followed by scintillation counting of the $^{45}\text{Ca}^{2+}$ trapped on the filters. Values are expressed as mean \pm SEM, and statistical significance was evaluated by using the Mann-Whitney U test (*, P , 0.05, control vs. PKA). (D). Micrographs showing Ca^{2+} -dependent loading of nuclear preparation with 10 kDa Calcium Green-1 dextran under control and PKA-phosphorylated conditions. For each condition, fluorescence ($\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 531 \text{ nm}$, Upper) and corresponding phase-contrast (Lower) micrographs are shown. Note that the dye fluorescence is seen at lower free calcium level once NCA is phosphorylated.

Table 4. Summary of partial purification of NCA

Purification step	Total protein, mg	Specific activity, fmol/mg per min	Total activity, fmol/min	Yield, %
Isolated nuclei	56	12.9	722	100
Supernatant	9.8	63	617	85.5
Peak I	0.17	2,020	343	47.5
Peak II	0.75	242.9	182.2	25.2

Ca²⁺-ATPase activity was determined by measuring Pi produced by ATP hydrolysis. For peak I (fractions 9-17 pooled) and peak II (fractions 24-36 pooled), partial purification was performed in the presence of 1 mM CaCl₂ and 2 mM phenylmethylsulfonyl fluoride, followed by gel-filtration of pooled fractions using Sephadex G-50 fine to eliminate AMP-PNP and NaCl. Blanks were estimated in the same way except that no protein was added, and values (expressed in fmol/mg per min) were corrected accordingly. Addition of trichloroacetic acid before addition of ATP yielded Pi counts equal to blanks. Basal ATPase activity (with 2 mM EGTA and without added calcium) was subtracted from ATP hydrolysis in calcium buffer to yield Ca²⁺-dependent ATPase activity (31).

NCA. Partial NCA purification was associated with increased stoichiometry, indicating the availability of additional sites for phosphorylation. The permeabilization of nuclei with digitonin did not alter the stoichiometry (0.30), therefore, it may be suggested that the phosphorylated sites are located on the part of the pump facing the cytoplasm. Alkaline phosphatase treatment before phosphorylation, enhanced the stoichiometry (0.81) of the membrane inserted NCA. This finding suggests that an endogenous level of phosphorylating activity, which targets the same sites as those targeted by PKA *in vitro*, operates *in vivo*. It may be argued that nearly all the NCA molecules are available for PKA phosphorylation, except a subpopulation (about 20 %) that may be PKA-insensitive NCA.

3.7. Effect of PKA phosphorylation of NCA on nuclear envelope Ca²⁺ transport activity

We examined the functional relevance of NCA phosphorylation by PKA by measuring its influence on ATP-dependent Ca²⁺ uptake (1). ATP-dependent nuclear ⁴⁵Ca²⁺-transport was Ca²⁺-dependent with a maximum at 1 μM free Ca²⁺ (Figure 14C). Free Ca²⁺ levels at which activation of the nuclear Ca²⁺-pump is maximum were relatively high; thus compatible with a role for NCA (and SERCA in general) in Ca²⁺ signaling through signal termination (59). Phosphorylation by PKA had a stimulating effect on Ca²⁺ uptake into purified nuclei, with maximum activity shifted to 2 μM free Ca²⁺ concentration in the medium.

Functional relevance of NCA phosphorylation by PKA was also investigated by measuring the transport of Calcium Green-1 10 kDa dextran, a fluorescent dye, into isolated rat liver nuclei (Figure 14D) and nuclei of cultured HEK293 cells (37). PKA phosphorylation of NCA enhances the transport of 10 kDa Calcium Green-1 dextran into the nucleus. This indicates the filling of the nuclear calcium pool regulating the opening of the nuclear pore complexes. This result is in agreement with the stimulatory effect of PKA phosphorylation on Ca²⁺ pumping activity into purified nuclei. The control of Ca²⁺-dependent NLS-independent macromolecular transport appears to be a novel function mediated by phosphorylation of the NCA by PKA (31).

No fluorescence in the nucleus was observed when isolated nuclei were incubated with 500 kDa Calcium Green-1 dextran (data not shown). ATP-dependent filling

of the Ca²⁺ store in the NE lumen was necessary for transport of the 10 kDa macromolecules. Phosphorylation by PKA accelerated this process (Figure 14D). The difference between transport into control and phosphorylated nuclei was statistically significant for pCa = 7.0 and pCa = 6.5.

3.8. Nuclear 1,3,4,5-tetrakisphosphate receptor (IP₄R)

Inositol 1,3,4,5-tetrakisphosphate (IP₄) is the most potent inhibitor of [³H]IP₄ binding to the isolated nuclei (Figure 15A) giving a biphasic response. For characterizing ligand binding, saturation techniques provide more precise evaluation than the commonly used isotopic dilution method. The biphasic nature of [³H]IP₄ binding to the nucleus was confirmed by Scatchard analysis of saturation isotherms (Figure 15B). A high affinity binding site, distinct from the low affinity one was characteristic of K_d = 1.6 nM and B_{max} = 1.25 pmol/mg protein. For high affinity, 3800 IP₄ binding sites were found per nucleus. This was the first observation for the presence of putative IP₄R on the isolated rat liver nuclei (23).

The distribution of the high and low affinity [³H]IP₄ binding sites were investigated by Scatchard analysis of saturation isotherms indicating that IP₄ binding sites were located both on the outer (Figure 16A) and on the inner (Figure 16B) nuclear membranes. This gave distinct K_d and B_{max}, corresponding to the high and low affinity binding sites observed with the intact nuclear preparation. Thus, the two types of binding sites were distinctly distributed on the two membranes of the NE, but they were not identical either quantitatively or qualitatively. We have located high affinity IP₄R to the outer nuclear membrane (Figure 16C) and the low affinity one located on the inner nuclear membrane (Figure 16D). The nature and function of low affinity IP₄R located on the nuclear membrane remains unexplored.

The purified nuclear IP₄R (Figure 17) was found to be a 74 kDa protein (26) and distinct from various other IP₄Rs with similar pharmacology. For instance, from pig cerebellum a protein of 42 kDa has been purified (28), from rat cerebellum proteins of 84, 174 and 182 kDa have been purified (28) and shown to be putative IP₄R; whereas platelets, which are anucleated cells, have 104 kDa protein as IP₄R (29). The 104 kDa platelets IP₄R appears to be a member of the family GTPase-activating proteins implicated in the activation of Ras (29). For unknown reasons the research on IP₄R remains almost unexplored.

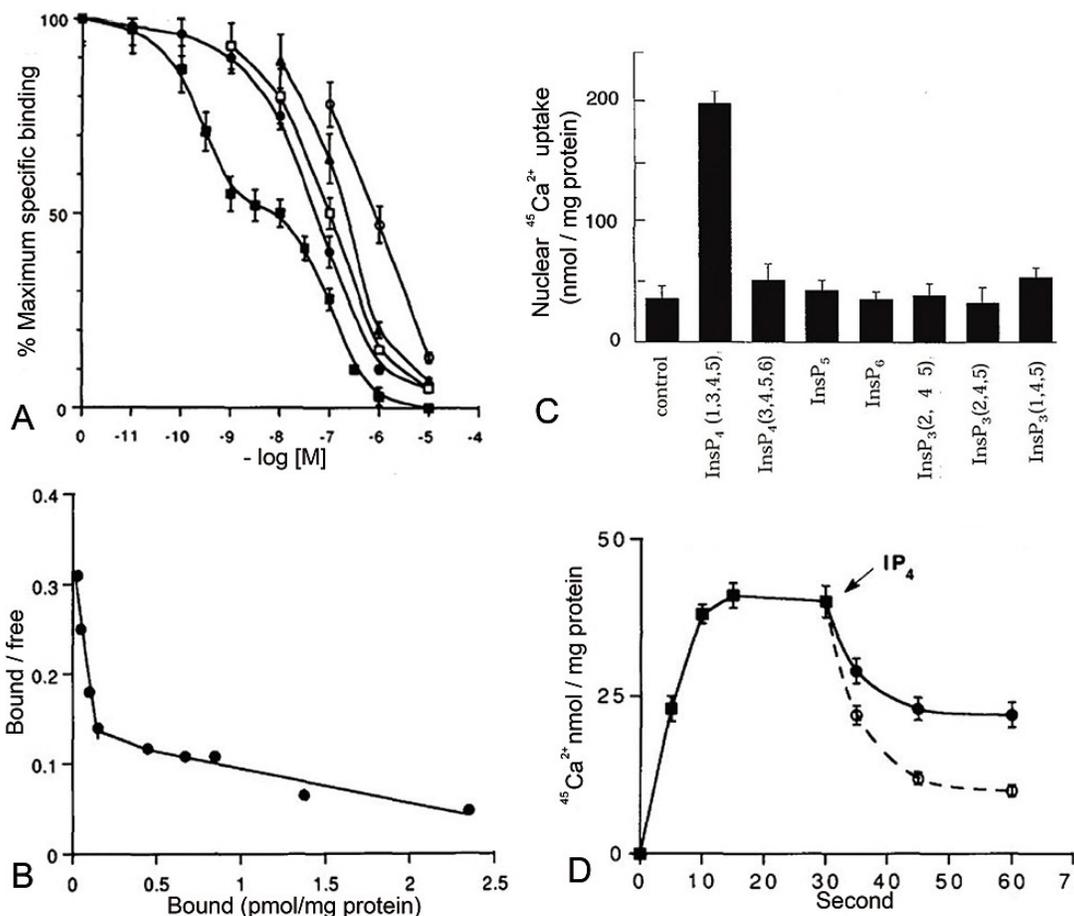


Figure 15. [³H]IP₄ binding to the rat liver nuclei and IP₄ role in nuclear Ca²⁺ movement. (A) Displacement of [³H]IP₄ binding by various concentrations of IP₄ (■), IP₅ (●), Ins(3,4,5,6) (□), IP₃ (▲), and adenosine 5'-tetrphosphate (○) is shown. The nonspecific binding was corrected in the presence of 10 μM IP₄. Concentration of [³H]IP₄ was 2 nM. (B) [³H]IP₄ binding was performed as described (72), employing varying concentrations of [³H]IP₄, from 0.1 to 100 nM. Scatchard analysis (23) was carried out under saturation binding conditions, i.e. by utilizing progressively increasing concentrations of [³H]IP₄. These experiments were performed on two independent nuclear preparations in quadruplicate. (C) Isolated nuclei were incubated at 37°C for 5 min in the presence of various inositol phosphates : control, IP₄(1,3,4,5) 100 nM, IP₄(3,4,5,6) 100 nM, IP₅ 100 nM, IP₆ 100 nM, IP₃(2, 4 5) 5 μM, IP₃(2,4,5) 100 nM, IP₃(1,4,5) 5 μM. Calcium chloride was supplemented into the medium bathing nuclei so as to give 1 μM free calcium. Traces of ⁴⁵Ca²⁺ (2.0 μCi/ml; 1 Ci = 37 GBq) were present. Calcium uptake was terminated by filtering under vacuum over GF/B Whatman glass fiber filters. ⁴⁵Ca²⁺ trapped on the filters was determined by spectrometry. Each experiment was carried out in quadruplicate. (D) IP₃-induced Ca²⁺ release (■) was performed as described (23), and after 30 s of IP₃ addition, IP₄ was added to give a final concentration of 1 nM (●) or 5 μM (○). At indicated times, the release was terminated by filtration under vacuum. IP₄-induced Ca²⁺ reuptake was defined as the radioactivity trapped after 30 s (steady state of IP₃-induced ⁴⁵Ca²⁺ release) minus the radioactivity trapped after IP₄ addition. These experiments were performed in quadruplicate on three independent nuclear preparations.

3.9. IP₄-mediated calcium transport

The role of IP₄R in nuclear calcium movement was the next most logical parameter to explore. The isolated nuclei, even in the absence of any added ATP, were capable of ⁴⁵Ca²⁺ uptake in the presence of IP₄ (Figure 15C). This indicated that the nuclei were endowed with an IP₄-dependent calcium transport system analogous to that operating in the secretory vesicles (60). The IP₄-mediated ⁴⁵Ca²⁺ uptake found further support by the IP₄-triggered reuptake of ⁴⁵Ca²⁺ that was initially released by IP₃ from the nuclei (Figure 15D). A comparison of nuclear calcium uptake by IP₄ observed in the absence and in the presence

of IP₃ indicated that IP₃ is able to potentiate the action of IP₄ (Figure 15D). The rate of calcium uptake by IP₄ in the absence and in the presence of IP₃ was 0.16 and 4.0 nmol/S/mg protein, respectively. Thus a role of IP₄ in nuclear calcium signaling is well founded (23, 24) and appears to be a dynamic phenomenon.

3.10. A holistic view emerges

Calcium is transported into the nucleus by the activation of NCA or IP₄R. But the route of exit (Figure 18) of calcium from the nucleus is through the nuclear pore complex (NPC). NPC is a large macromolecular assembly

Inositol and calcium signals in the nucleus

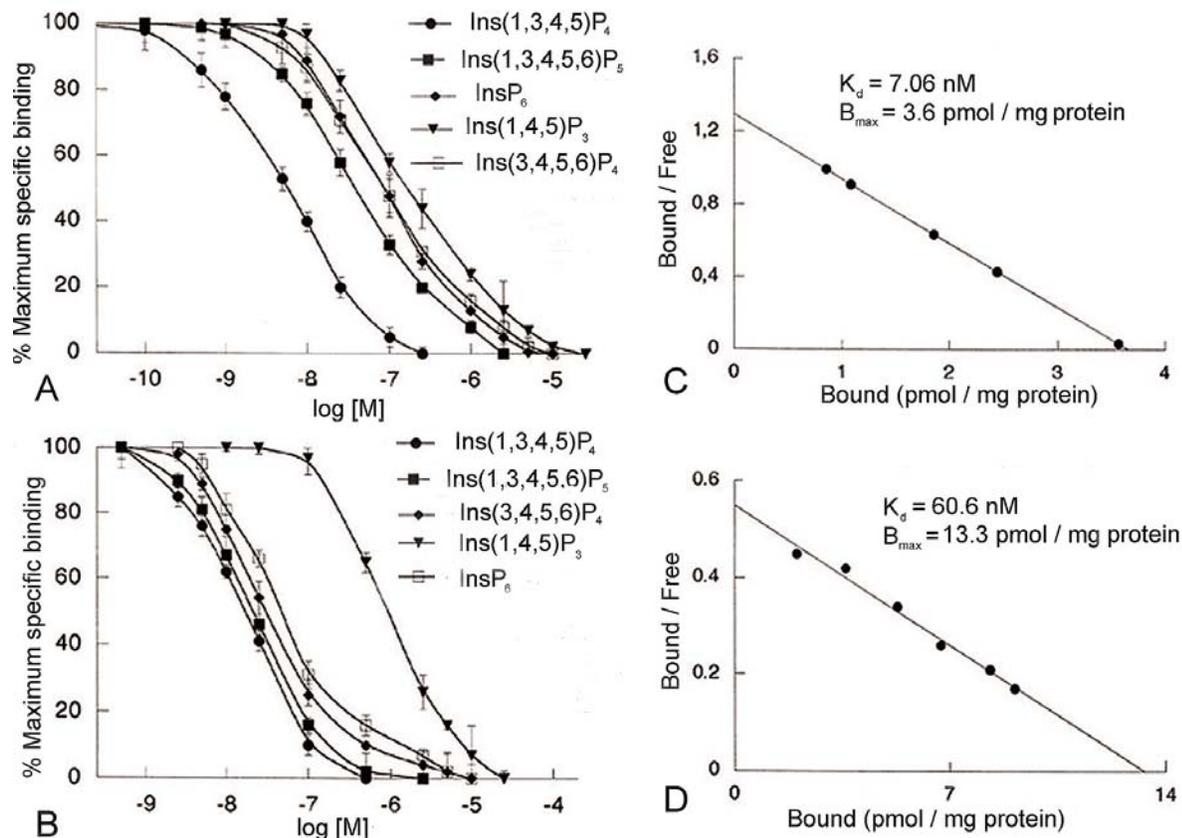


Figure 16. Selective displacement of [^3H]IP $_4$ binding to the outer (A) and inner (B) nuclear membrane. The purified membranes were used, and the binding assay was carried out in an Eppendorf tube for 10 min at 0°C (on ice) in a final volume of 400 μl . Details of binding conditions are described (25). Nonspecific binding was determined in the presence of 10 μM nonradioactive inositol phosphates. Bound and free radioligands were separated by centrifugation. [^3H]IP $_4$ concentration was 1 nM. Scatchard plots of [^3H]IP $_4$ binding to the outer (C) and inner (D) nuclear membrane. These experiments were carried out progressively increasing concentrations of [^3H]IP $_4$. Two independent membrane preparations were used for this study. Each experiment was done in quadruplicate, with replicates varying by <10%.

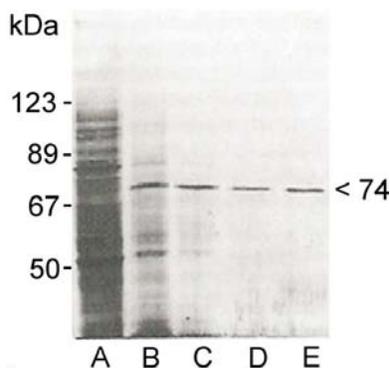


Figure 17. SDS-PAGE profile at various purification steps of nuclear IP $_4$ R. Proteins obtained after each step of purification (26) were loaded on a 1.5 mm thick SDS-polyacrylamide gel (T% = 8.1 ; C% = 3). Electrophoresis was carried out at 25 mA constant current, and proteins were silver stained. Each lane represent a step of purification : (lane A) solubilized nuclear protein (first step) ; (lane B) protein pooled after first isoelectric focusing (second step) ; (lane C) protein pooled after refocusing (third step) ; (lane D) protein pooled after the electrophoretic mobility step (last but one step) ; and (lane E), protein obtained after electroelution from a semipreparative gel under nondenaturing conditions (final product, i.e. purified nuclear receptor). Numbers on the left-hand side indicates molecular mass in kDa. On the right hand side arrow indicates 74 kDa nuclear IP $_4$ R. The amount of protein loaded on each lane was 0.1 – 1.0 μg .

Inositol and calcium signals in the nucleus

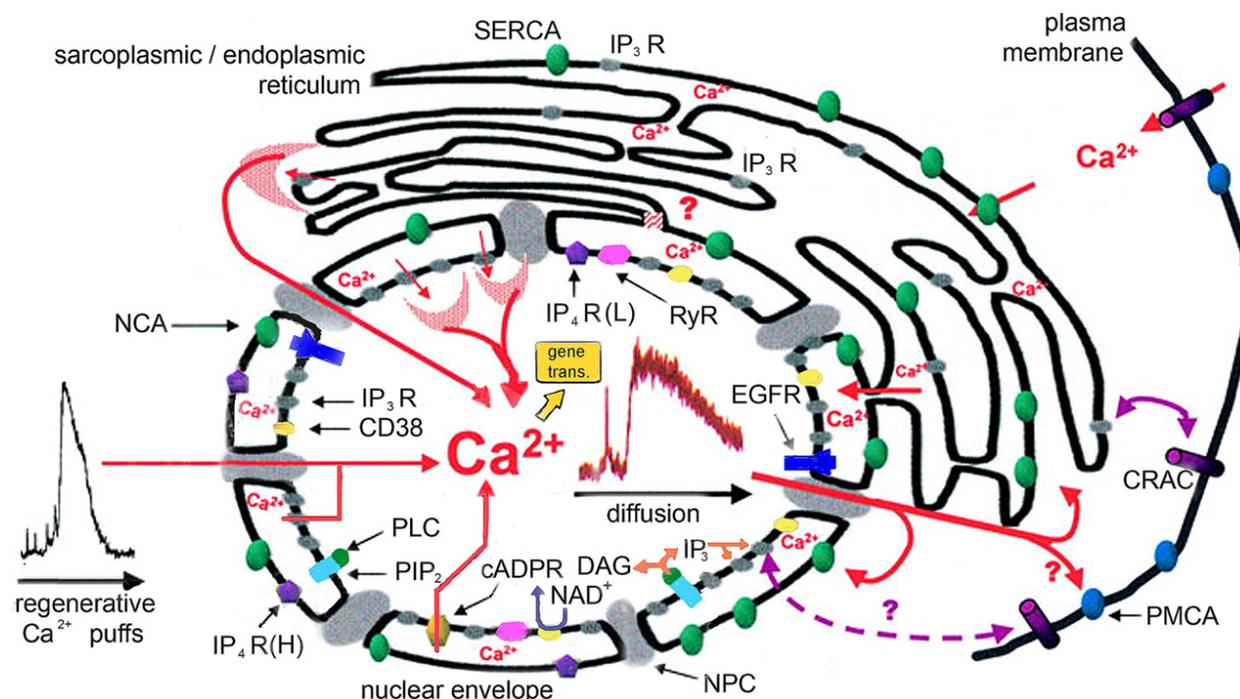


Figure 18. Dynamics of nuclear calcium signaling. The entry of calcium into the nucleus is a two stage process. Nuclear envelope calcium pool which serves as a transient calcium store is filled either with NCA or IP₃R-located on the outer nuclear membrane (73). But the calcium release from the NE into the nucleoplasm is carried out either with IP₃R or RYR-located on the inner nuclear membrane (25, 32). Nuclear IP₃ receptors could be involved in the capacitative influx of Ca²⁺. cADP-ribose is generated by the action of CD38/ADP ribosyl cyclase (50) on NAD, and serves as a ligand for RYR. IP₃ is produced in the nucleus by the EGF nuclear signaling activating nuclear membrane located EGFR and consequently stimulating nuclear PLC-gamma 1. NCA and IP₃R are also activated by serine phosphorylation by PKA and PKC located in the nucleus, respectively. Whereas PLC-gamma 1 is stimulated by the tyrosine phosphorylation and its consequent binding with the EGFR. Thus NE calcium pool can also be filled by PKA phosphorylation of NCA (31, 37). External free calcium concentrations play a dominant role in 'on' and 'off' mechanism of NCA activation and IP₄R operation (see discussion). Cytosolic Ca²⁺ 'puffs' generated in the immediate perinuclear vicinity of the NE – at the mean distance of less than 4 – 6 μm are instantly transmitted to the nucleus. Ca²⁺ 'puffs' produced at distances greater than 6 μm from NE are not transmitted (74) to the nucleus. Thus the cytosolic Ca²⁺ rises do not reach the nucleus on its own and require NCA or IP₄R. The Ca²⁺ is dissipated by egression through the NPC from its nucleoplasmic face (64) followed by sequestration into the ER.

that spans the inner and outer nuclear membranes (61). The nucleoplasmic site of the NPC architecture is totally different from that of its cytoplasmic site (62, 63). The opening of the NPC at the nucleoplasmic face in the presence of 1 μM Ca²⁺ has been studied in 'unfixed' isolated NPC in a physiological buffer (64) by Atomic Force Microscopy.

A picture of nuclear calcium signaling that emerges here is schematically illustrated in Figure 18. The nucleus is endowed, in its own right, all the essential components implicated in nuclear calcium signaling, including phospholipases and lipids (65, 66). The eukaryotic nucleus is separated from the cytoplasm by the NE. The NE comprises two membranes bilayers, the inner and outer nuclear membranes, which are separated by a lumen. The two nuclear membranes join periodically to one another at the sites where NPCs are embedded. Ca²⁺-ATPase and IP₄R are located on the outer nuclear membrane, act to fill the nuclear envelope calcium pool. Calcium is released from the NE into the nucleoplasm

when nuclear generated IP₃ binds IP₃R located on the inner nuclear membrane. The PLC-gamma 1 is activated through its phosphorylation (44) by nuclear membrane located EGFR (18) at the tyrosine and thus hydrolyses PIP₂ producing IP₃ and DAG. cADP ribose is produced in the nucleus by the activation of CD38/ADP ribosyl cyclase (50), located on the inner nuclear membrane, targeting NAD. cADP ribose serves as a ligand for the ryanodine receptor (32, 33, 50), which is also located on the inner nuclear membrane.

The scheme outlined in this review (Figure 18) delivers a holistic concept of nuclear calcium signaling. The tyrosine phosphorylation of PLC-gamma 1 and its consequent activation generating nuclear IP₃ establishes complementarity with the serine phosphorylation of IP₃R in regulating nuclear calcium homeostasis. This pathway of calcium availability in the nucleoplasm is regulated by the filling of the nuclear envelope calcium pool which is under regulation of NCA phosphorylation at the serine by PKA. Evidence are also advanced in this review that partial

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proteolysis constitutes an important paradigm in nuclear signal transduction pathway. Although the mechanism of nuclear proteolysis remains a virgin area for future exploration.

Thus an autonomous nuclear calcium regulation (11, 39, 43) and increases in the nuclear calcium levels carrying specific functions that are distinct from the effect of an increase in the cytosolic calcium level (67-70) are profoundly supported by the mechanism of calcium signal in the nucleus proposed here.

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Abbreviations : NCA : Nuclear Ca^{2+} -ATPase, RYR : ryanodine receptors, IP_3 : inositol 1,4,5-trisphosphate, IP_3R : inositol 1,4,5-trisphosphate receptor, PLC : Phospholipase C, PIP_2 : phosphatidylinositol-4,5-bisphosphate, DAG : diacylglycerol, PIKE : phosphatidylinositol-3-OH-kinase enhancer, PI(3)K : phosphatidylinositol-3-OH-kinase, EGF : epidermal growth factor, EGFR : epidermal growth factor receptor, 3,4,5- IP_3 : inositol 3,4,5-trisphosphate, PKC : protein kinase C, IP_4 : inositol 1,3,4,5-tetrakisphosphate, IP_4R : inositol 1,3,4,5-tetrakisphosphate receptor, IP_6 : Inositol hexakisphosphate, PKA : cAMP-dependent protein kinase, dbc-AMP : dibutyryl cyclic AMP, ($[^3H]$)PI : ($[^3H]$ -phosphatidylinositol), SCOP : suprachiasmatic nucleus circadian oscillatory protein, NPC : nuclear pore complex, NE : nuclear envelope, CRAC : Ca^{2+} -release activated channel.

Key Words : Nucleus, Nuclear Envelope, Nuclear Signal, Nuclear Calcium, nuclear calcium-ATPase, Nuclear Protein Kinase C, Cyclic AMP-dependent Protein Kinase, Serine Phosphorylation, Tyrosine Phosphorylation, Protein Phosphorylation, Nuclear Inositides, Inositol Phosphates, Inositol Trisphosphate, Inositol Trisphosphate Receptor, Inositol Tetrakisphosphate Receptor, Epidermal Growth Factor, Epidermal Growth Factor Receptor, Ryanodine Receptor, Phospholipase C-gamma 1, Phosphatidylinositol-3-OH-Kinase enhancer, phosphatidylinositol-3-OH-Kinase,

diacylglycerol, Review

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