Rat Inositol 1,4,5-Trisphosphate 3-Kinase C Is Enzymatically Specialized for Basal Cellular Inositol Trisphosphate Phosphorylation and Shuttles Actively between Nucleus and Cytoplasm

Marcus M. Nalaskowski‡, Uwe Bertsch‡, Werner Fanick‡, Malte C. Stockebrand‡, Hartwig Schmale§, and Georg W. Mayr‡¶

From the §Institute for Cellular Signal Transduction and the ¶Institute for Cell Biochemistry and Clinical Neurobiology, University Hospital Hamburg-Eppendorf, Martinistrasse 62, 20246 Hamburg, Germany

The calcium-liberating second messenger inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) is converted to inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P4) by Ins(1,4,5)P3 3-kinases (IP3Ks) that add a fourth phosphate group to the 3-position of the inositol ring. Two isoforms of IP3Ks (named A and B) from different vertebrate species have been well studied. Recently the cloning and examination of a human full-length cDNA encoding a novel isoform, termed human IP3K-C (HsIP3K-C), has been reported. In the present study we report the cloning of a full-length cDNA encoding a rat homologue of HsIP3K-C with a unique mRNA expression pattern, which differs remarkably from the tissue distribution of HsIP3K-C. Of the rat tissues examined, rat IP3K-C (RnIP3K-C) is mainly present in heart, brain, and testis and shows the strongest expression in an epithelial tissue, namely tongue epithelium. RnIP3K-C has a calculated molecular mass of ~74.5 kDa and shows an overall identity of ~75% with HsIP3K-C. A bacterially expressed, enzymatically active and Ca2+-calmodulin-regulated fragment of this isoform displays remarkable enzymatic properties like a very low Kₘ for Ins(1,4,5)P₃ (~0.2 μM), substrate inhibition by high concentrations of Ins(1,4,5)P₃, allosteric product activation by Ins(1,4,5)P₃ in absence of Ca2+-calmodulin (Kₐ(app) 0.52 μM), and the ability to efficiently phosphorylate a second InsP₃ substrate, inositol 2,4,5-trisphosphate, to inositol 2,4,5,6-tetakisphosphate in the presence of Ins(1,3,4,5)P₄. Furthermore, the RnIP3K-C fused with a fluorescent protein tag is actively transported into and out of the nucleus when transiently expressed in mammalian cells. A leucine-rich nuclear export signal and an uncharacterized nuclear import activity are localized in the N-terminal domain of the protein and determine its nucleocytoplasmic shuttling. These findings point to a particular role of RnIP3K-C in nuclear inositol trisphosphate phosphorylation and cellular growth.

For the conversion of the calcium-liberating second messenger inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) (1), all metazoan cells examined so far seem to possess two types of enzyme activities: first, the 5-phosphatases (2), which degrade it to inositol 1,4-bisphosphate and thereby initiate the recycling of the inositol moiety into membrane phospholipids; and second, the 3-kinases (IP3Ks) (2), which use ATP to add a fourth phosphate group to the 3-position of the inositol ring to generate the signaling molecule inositol 1,3,4,5-tetrakisphosphate (Ins (1,3,4,5)P₄). This InsP₄ isomer has been implicated in signaling functions concerning the regulation of a Ras GTPase-activating protein (3), isosteric inhibition of Ins(1,4,5)P₃ 5-phosphatases (30) and thus prolongation of Ins(1,4,5)P₃ signals, as well as calcium entry through the plasma membrane (4), and other potential functions (5, 6). Moreover, Ins(1,3,4,5)P₄ is the first product derived from Ins(1,4,5)P₃ that can be converted to a plethora of inositol phosphate isoforms found in metazoan cells (7), ranging from different InsP₃ isoforms through InsP₅ to InsP₇. This isoform displays remarkable enzymatic properties like a very low Kₘ for Ins(1,4,5)P₃ (~0.2 μM), substrate inhibition by high concentrations of Ins(1,4,5)P₃, allosteric product activation by Ins(1,4,5)P₃ in absence of Ca2+-calmodulin (Kₐ(app) 0.52 μM), and the ability to efficiently phosphorylate a second InsP₃ substrate, inositol 2,4,5-trisphosphate, to inositol 2,4,5,6-tetakisphosphate in the presence of Ins(1,3,4,5)P₄. Furthermore, the RnIP3K-C fused with a fluorescent protein tag is actively transported into and out of the nucleus when transiently expressed in mammalian cells. A leucine-rich nuclear export signal and an uncharacterized nuclear import activity are localized in the N-terminal domain of the protein and determining its nucleocytoplasmic shuttling. These findings point to a particular role of RnIP3K-C in nuclear inositol trisphosphate phosphorylation and cellular growth.

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function (9), has been reported. Dewaele et al. (10) reported that the HsIP3K-C is a predominantly cytosolic enzyme when transiently expressed in COS-7 cells, but did not demonstrate a specific targeting function of its N-terminal domain. Thus, the genuine function of IP3K-C and the regulatory or targeting function of its N-terminal domain remained unclear up to now.

In this study we highlighted possible special roles of IP3K-C in the cellular and in particular in nuclear inositol phosphate metabolism. A rat homologue of HsIP3K-C, termed rat Ins(1,4,5)P_3-kinase C (RnIP3K-C) was identified. Its mRNA expression pattern in different tissues, the enzymatic properties and the allosteric regulation of bacterially expressed enzyme, and the nucleocytoplasmic shuttling of EGFP fusion proteins derived from RnIP3K-C expressed in mammalian cells were examined. The most interesting findings of our study are that the RnIP3K-C enzyme is strongly Ca^{2+}-CaM-activated in a substrate concentration-dependent manner, is enzymatically optimized for an efficient conversion of basal cellular concentrations of Ins(1,4,5)P_3 in presence of Ins(1,3,4,5)P_4, and can convert Ins(2,4,5)P_3 to Ins(2,4,5,6)P_4 under these conditions. Furthermore, RnIP3K-C undergoes active nucleocytoplasmic shuttling as a result of a hitherto unidentified nuclear localization activity and an identified nuclear export signal (NES) both residing in the N-terminal domain of the enzyme. Active transport of proteins between the nucleus and cytoplasm is mediated mainly by the intensively studied canonical nuclear localization signals (NLS) and by the recently characterized nuclear export signal (14). By employing LMB inhibition and deleting the canonical NES in the N-terminal domain of RnIP3K-C, we could prove the functionality of this site and the nuclear shuttling activities of the enzyme when expressed in cells.

**EXPERIMENTAL PROCEDURES**

**Cloning of Rat Inositol 1,4,5-Trisphosphate 3-Kinase Isoform C (RnIP3K-C)**

cDNA—Degenerate primers (forward, 5'-CATGCTTGCTGGTGCATGAG-3' and reverse, 5'-AGGCC(G/A)TGCTGC(A/T)GCTCCG-3') were designed according to the completely conserved amino acid sequences KPRYMQW and EGNREDG, respectively, in the catalytic domain of rat and human IP3K-A and IP3K-B, and IP3K from chicken (see Ref. 9 and citations within). A PCR using 3 μl of a LZAP Express cDNA library prepared from rat circumvallate papilla (16) as template in a total volume of 50 μl was carried out as follows: 94 °C for 3 min, 40 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 10 min. PCR products isolated from the single band of ~400 bp were subcloned into pUC18 using the SureClone ligation kit (Amersham Biosciences). The nucleotide sequences were determined by the dye terminon method with an ABI Prism 373A sequence analyzer (Applied Biosystems, Foster City, CA). 22 pluge pools of the circumvalitate cDNA library, each consisting of ~5 × 10^6 independent plugging fragments, were screened using the PCR with the primer pair and under conditions as described above. 1 × 10^5 plaque-forming units of each of the three pools that were positive in the PCR pre-selection were plated onto Escherichia coli XL-1 MR' cells; the DNA was transferred to nitrocellulose filters and hybridized conventionally with the 32P-labeled subcloned PCR fragment. The hybridizing 11 plaques were purified by three rounds of plating and hybridization and finally converted into recombinant pBK-CMV plasmids by in vivo excision according to the protocol from the supplier (Stratagene, La Jolla, CA). After diagnostic restriction fragment analysis, the clone exhibiting the longest insert of ~3.3 kb was sequenced on both strands using a set of gene-specific primers.

**5'-RACE—mRNA, purified by oligo(dT) columns (Amersham Biosciences) from total RNA prepared from rat brain (strain Wistar) as described (17), was used for 5'-RACE employing the Marathon™ kit (Clontech) according to the instructions from the manufacturer. For reverse transcription, carried out for 1 h at 42 °C, a gene-specific primer (AGTGCCCTGCTGGTCATGAG-3') was used in a reaction containing 3.5 μg of mRNA. With the generated double-stranded cDNA, a first round of PCR was performed using a gene-specific primer (5'-GGCCA- CCGGCGGCCGCTCATC-3') and primer AP1 (Marathon™ kit) with an annealing temperature of 56 °C. On the diluted PCR product, nested PCR was performed using a gene-specific primer (5'-CTTCCCTTTA- ATGTACCA-3') and primer AP2 (Marathon™ kit) and an annealing temperature of 56 °C. A second round of RACE PCR was done on the original cDNA template with a gene-specific primer (5'-GCTCCA- GGGCGGCTGATGATGAC-3') and primer AP1 using an annealing temperature of 56 °C. From this reaction a nested PCR was performed with a gene-specific primer (5'-TATGAGGGGTATACCCGT-3') and primer AP2 at 59 °C and annealing temperature of 56 °C.

**Northern Blot Analysis—** A Northern blot containing ~2 μg of polyA⁺ RNA/lane from eight different rat tissues was purchased and hybridized according to the instructions from the supplier (Clontech). The 3.3-kb DNA fragment representing the full-length RnIP3K-C cDNA labeled with [³²P]dCTP by random priming was used for hybridization in ExpressHyb solution at 68 °C for 90 min, followed by washing two times in 0.1× SSC, 0.1% SDS for 40 min at 50 °C.

To compare specifically RnIP3K-C mRNA in tongue and taste epithelium with other tissues, 15 μg of glyoxylated total RNA/lane was separated by agarose gel electrophoresis, transferred onto a Hybond N membrane (Amersham Biosciences) and hybridized with the radiolabeled full-length RnIP3K-C probe described above. Hybridization was performed in 20× SSC at 42 °C overnight and washing in 0.2× SSC, 0.1% SDS at 45 °C were carried out according to the instructions from the manufacturer (Ambion, Austin, TX). As control for RNA loading and integrity, the blot was stripped and hybridized with a 32P-labeled actin probe under the same conditions. Blots were exposed to a phosphorimaging screen for up to 24 h and analyzed in a Fujix Bio-Imaging Analyzer (LAS 2000).

**Reverse Transcription-PCR—** 1 μg of total RNA from various rat tissues including enzymatically prepared tongue and taste epithelium was reverse transcribed using Superscript II reverse transcriptase (In-vitrogen) and random hexamer primers (Amersham Biosciences). A pair of RnIP3K-C-specific primers (forward, 5'-TTCACCAGCCTTCCTC-3' and reverse, 5'-GAAGCCATCGGTGTCACTGG-3') was designed to amplify a 271-bp fragment from 1/20 of the reverse transcribed total RNA using the Taq PCR Master Mix (Qiagen). PCR was performed in 25 cycles (94 °C, 1 min for the initial denaturation, 94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s for each cycle and 72 °C, 10 min for the final elongation). Following the same protocol, a hypoxanthine-guanine-phosphoribosyltransferase fragment of 526 bp was amplified as control. PCR products were visualized with ethidium bromide after separation of equal volumes of each reaction on a 1.8% agarose gel.

**Bacterial Expression of a RnIP3K-C Fragment Comprising the CaM Binding and the Catalytic Domain (CBD-RnIP3K-C) and a Fragment Comprising Only the Catalytic Domain (ΔCBD-RnIP3K-C)—** A fragment of the protein coding region of RnIP3K-C (termed CBD- RnIP3K-C) comprising CaM binding and catalytic domain (amino acids 366–678) was amplified with the following primer pair (5'-GGGAT- GCCATATGTCGTCGGGGGCGGTTACAG-3'; 5'-CTCTACTAGT- GAGGCTAGTGGGCTAGCG-3') in a PCR (25 cycles) using 2.5 units of Pfu polymerase (Promega) in a 100-μl standard reaction mixture according to the manufacturer (annealing temperature of 65 °C for 4 min and 65 °C melting down at 72°C/Cycle). The PCR product was first cloned into the pGEM T-Easy vector (Promega). The cloned PCR product was cleaved out with NdeI and SpeI and re-ligated into pET17b vector (Novagen) previously cut with the same restriction enzymes. The re-cloned pET17b fragment of 526 bp was transformed in E. coli BL21(DE3)pRIL cells (Novagen) transformed with the resulting expression vector and purified by phosphocellulose and CaM affinity chromatography essentially as described previously for chicken IP3K (9).

A fragment of RnIP3K-C (termed ΔCBD-RnIP3K-C) comprising only the catalytic domain (amino acids 410–678) was amplified with the following primer pair (5'-GGGATCCCATATGTCGGTCGGGGGCGGTTACAG-3'; 5'-CTCTACTAGT-GAGGCTAGTGGGCTAGCG-3') and a fragment of 200 bp polymerase (Promega) in a 100-μl standard reaction mixture according to the manufacturer (annealing temperature of 65 °C for 4 min and 65 °C melting down at 72°C/Cycle). The PCR product was first cloned into the pGEM T-Easy vector (Promega). The cloned PCR product was cleaved out with NdeI and SpeI and re-ligated into pET17b vector (Novagen) previously cut with the same restriction enzymes. The resulting recombinant plasmid pET17b-RnIP3K-C was transformed in E. coli BL21(DE3)pLysS,pREP cells (Novagen) previously cut with the same restriction enzymes. The resulting recombinant plasmid pET17b-RnIP3K-C was transformed in E. coli BL21(DE3)pLysS,pREP cells (Novagen) previously cut with the same restriction enzymes. The resulting recombinant plasmid pET17b-RnIP3K-C was transformed in E. coli BL21(DE3)pET17b vector. The resulting recombinant plasmid pET17b-RnIP3K-C was transformed in E. coli BL21(DE3)pET17b vector.
Enzymatic Analysis of CBD-RnIP3K-C and ΔCBD-RnIP3K-C—Enzymatic activities were measured under various substrate, product, activator, and inhibitor concentrations using a coupled enzymatic optical assay essentially as described previously (9). A Lambda 20 UV-visible spectrometer (PerkinElmer Life Sciences) equipped with thermostatted cuvette holder and numerical data storage and derivation device (program UV Winlab, PerkinElmer Life Sciences) was employed. The pH 7.5 reaction conditions used in the assays were: 10 mM triethanolamine, pH 7.5, 5.5 mg MgCl₂, 30 mM KCl, 1 mM dithiothreitol, 0.2 mM NADH, 1 mM phospho(enol)pyruvate, 0.5 mM ATP, 5 units/ml lactate dehydrogenase, 2.5 units/ml PK in the presence of 6 mM ammonium sulfate. All assays were performed at 30 °C after a 15-min preincubation of the reaction mixture. After following background NADH consumption (for a determination of PK, lactate dehydrogenase, the reaction was followed for 5–10 min), the IP3K assay was started with inositol phosphate substrate. All assays were performed in absence and presence of 0.1 μM Ca²⁺-CaM. In the latter case, 20 μM Ca²⁺ was present. The dependence of enzymatic activity on the concentration of Ins(1,4,5)P₃ (bought from Alexis, Woburn, MA) was assayed by single transients. They were started at differing initial InsP₃ concentrations (varying between 1 and 35 μM) and followed in the optical assay until InsP₃ was completely consumed. The Kₘ for ATP was assayed by varying the initial ATP concentration at a fixed initial Ins(1,4,5)P₃ concentration of 5 μM and measuring initial activities. Activation by Ca²⁺-CaM was also assayed by measuring initial activities at 5 μM initial Ins(1,4,5)P₃ concentration and that of CaM was varied. Activation or inhibition by Ins(1,3,4,5)P₄ was assayed by determining the initial enzyme activity at 1 μM Ins(1,4,5)P₃ and increasing concentrations of Ins(1,3,4,5)P₄ present before starting the optical assays. In case of the enzyme form ΔCBD-RnIP3K-C, the true Kₘ for Ins(1,4,5)P₃ of this enzyme form was determined from the Kₘ of ΔCBD-RnIP3K-C and the activity of active ΔCBD-RnIP3K-C minus the activity of inactive ΔCBD-RnIP3K-C. The y intercept of the resulting linear regression curve corresponds to Kₘ, the x intercept to −Kₘ for Ins(1,3,4,5)P₄. The reaction products generated by conversion of Ins(2,4,5)P₃ (pure synthetic Ins(2,4,5)P₃ was a gift from Barry V. L. Potter) and from assays of the activation of IP3K-C by Ca²⁺-CaM were analyzed by metal dithioester detection (MDD)—HPLC (18–20). For that, aliquots from the optical assay mixture were trichloroacetic acid-precipitated and charcoal-treated to remove ATP and NADH as described (18–20).

Construction of Fusion Genes and Fusion Gene Derivatives—The EGFP fusion variants N-tagfull and C-tagfull were created by PCR techniques. The open reading frame of RnIP3K-C was amplified using primer pairs (N-tagfull): 5'-GGCGCATAGGAGGCTCTCCGCCG-3' and 5'-GGATCCTATGGGTGATCTCCGAGCG-3'; C-tagfull: 5'-AGCGCTATGCAGAGGCTCTCCGAGC-3' and 5'-CTCGAGCTCGAGGCAAGGAG-3'. The PCR products were initially cloned into the pGEM Easy vector (Promega, Mannheim, Germany). The open reading frame was then subcloned, and the cloned PCR fragment was used as a template to extend the sequence further to the 5'-untranslated region (5'-UTR). The 5'-UTR sequence was confirmed independently by analysis of RT-PCR fragments that span the junction between the RACE products, two in-frame stop codons and no further sequence was used to screen a circumvallate papilla cDNA library under stringent conditions, a 3309-bp cDNA clone containing a single long open reading frame and a poly(A) tail of 18 bp was isolated. The translated protein consists of 678 amino acids with a predicted molecular mass of 74,463 Da. Because the first methionine residue was not preceded by an in-frame translational stop codon, 5'-RACE experiments were performed to extend the sequence further to the 5' end of the mRNA. In the 246 bp of sequence added by the analysis of the 5'-RACE products, two in-frame stop codons and no further in-frame initiation codon were identified. The presence of the 5'-sequence was confirmed independently by analysis of RT-PCR products that span the junction between the RACE products and the original cDNA clone (data not shown). Therefore, the novel sequence has 353 bp of 5'-untranslated region and the 5'-most ATG found in the cDNA clone most probably represents the initiation codon, although the neighboring residues do not agree well with the Kozak consensus sequence (from −9 to +4: [GCC/GCA/G/CATGG] (23)). The 3'-untranslated region of 1146 bp exhibits the polyadenylation signal AATAAA 18 nucleotides upstream of the poly(A) tail. The deduced protein sequence of RnIP3K-C shows significant identity with other IP3Ks and essentially the same domain structure (Fig. 1). The highest overall identity of −75% is found with the HsIP3K-C (10). Although the amino acid identities in the more conserved catalytic, calmodulin binding, and PEST do-
The Rat Inositol 1,4,5-Trisphosphate 3-Kinase C (RnIP3K-C) is expressed in a tissue-specific fashion at relatively low abundance indicated by the fact that only poly(A)⁺ RNA blots produced reasonable strong signals after overnight exposure. A single transcript of 3.4 kb was present predominantly in heart, brain, and testes; at lower levels in lung, liver, and kidney; and almost undetectably in spleen and skeletal muscle (Fig. 2A). The size of the transcript is consistent with the length of the cloned cDNA. The expression pattern of RnIP3K-C mRNA in rat tissues differs remarkably from that found in human organs (10). In humans, skeletal muscle showed highest expression, whereas brain and kidney contained almost no specific mRNA. Specific rat tissues such as tongue and taste epithelium exhibited amounts of RnIP3K-C mRNA that were considerably higher than those found in, e.g., rat brain (Fig. 2B). In these epithelial tissues, the 3.4-kb transcript could be detected on Northern blots even when using total RNA. The relatively high abundance of RnIP3K-C mRNA in taste and tongue epithelium compared with other tissues was confirmed by semiquantitative RT-PCR with a set of cDNAs prepared separately (Fig. 2C). The RT-PCR data correlate exactly with the results of both Northern blot experiments.

### Enzymatic Activities and Calmodulin Regulation of Bacterially Expressed RnIP3K-C Fragments—

Measurements of the enzymatic activity of a recombinant RnIP3K-C fragment comprising the catalytic and calmodulin binding domains (CBD-RnIP3K-C) revealed an unexpected behavior of this enzyme regarding its dependence on the substrate concentration. The enzymatic activity of the fragment is already very high at low Ins(1,4,5)P₃ concentrations (<100 nM), displays a maximum at ~0.3 μM, and decreases with higher substrate concentrations. This substrate inhibition effect is particularly evident in the absence of the activator Ca²⁺-CaM, whereas its presence leads to a higher relative activity at high substrate concentrations, i.e., a lower relative substrate inhibition. This has the consequence that the degree of Ca²⁺-CaM activation increases from ~4-fold at 0.3 μM Ins(1,4,5)P₃ to ~8-fold at 5 μM Ins(1,4,5)P₃ (Fig. 3, A and B). These data sets can be fitted almost equally well with a simplified model for general non-competitive substrate inhibition or a model for an ordered Bi-Bi reaction (for parameters and explanations, see subscripts of Fig. 3 (A and B), and Ref. 47). Only in the case without Ca²⁺-CaM does the ordered Bi-Bi reaction model seem to fit slightly better to the data points (Fig. 3A). As compared with the known Kᵥ values of IP3Ks ranging from 0.7 to 3.1 μM (see Ref. 29 and citations therein), depending on the source and isoform examined, very low Kᵥ values for Ins(1,4,5)P₃ of ~0.2 μM (0.15 or 0.27 μM, depending on the model employed for parameter derivation) in absence and ~0.18 μM (almost independent on the model employed) in presence of Ca²⁺-CaM were derived. Apparent Vₐₙₐₓ values of 2.5–3.0 units/mg in absence and 12 units/mg in presence of Ca²⁺-CaM (observed at ~0.3 and 0.5 μM Ins(1,4,5)P₃, respectively) could be derived from inspection of the v versus S curves in Fig. 3(A and B, respectively). These maximum values are lower than the “true” Vₐₚ maximum values derived for the reaction models (see parameters to Fig. 3 (A and B)) as a result of the substrate inhibition phenomenon. A Kᵥ value for the binding of and activation by Ca²⁺-CaM of 9.4 nM was determined at 5 μM Ins(1,4,5)P₃ and 0.5 mM ATP by the Ca²⁺-CaM-induced enzyme activation data (Fig. 3C). The Kᵥ value for ATP, determined at 5 μM Ins(1,4,5)P₃, was 33 μM in the absence and 52 μM in the presence of 0.1 μM Ca²⁺-CaM (data not shown).

Unusual is the effect that the product Ins(1,3,4,5)P₄ exerts on the enzyme in absence of Ca²⁺-CaM. Although both IP3K-A and IP3K-B show a marked competitive inhibition by Ins(1,3,4,5)P₄ with respect of the substrate Ins(1,4,5)P₃ in both absence and in presence of Ca²⁺-CaM (our own data for IP3K-B and IP3K-A); Ref. 29 for IP3K-A), the rat C-isoform displays a distinct activation by Ins(1,3,4,5)P₄ only in the absence of Ca²⁺-CaM. The degree of activation by Ins(1,3,4,5)P₄ determined at 1 μM Ins(1,4,5)P₃ is more than 2-fold, and an activation plateau is reached at ~3 μM Ins(1,3,4,5)P₄. An apparent Kᵥ for the binding of and activation by Ins(1,3,4,5)P₄ of 0.52 μM was derived from the data by assuming simple hyperbolic binding kinetics (Fig. 4A). MDD-HPLC analysis showed that no Ins₅P₃ product was formed in presence of Ins(1,3,4,5)P₄ (data not shown); therefore, the product activation observed is a true allosteric activation of the conversion of Ins(1,4,5)P₃. This allosteric effect is apparently abolished by Ca²⁺-CaM binding (Fig. 4B), which per se activates the enzyme.

To test the hypothesis that the product activation that is not observed in presence of Ca²⁺-CaM is really a regulatory property residing in the CaM binding domain of IP3K-C and is caused by binding of Ins(1,3,4,5)P₄ to this domain in the absence of Ca²⁺-CaM, we constructed a bacterial expression vector termed ΔCBD-RnIP3K-C, lacking the cDNA sequence coding for the CaM binding domain. This enzyme showed a more than 10-fold increased maximal specific activity in absence of Ca²⁺-CaM as compared with the enzyme containing the CaM binding domain (Vₐₚ max = 31 (± 1.5) units/mg versus Vₐₚ max(app) = 2.5 (± 0.8) units/mg) and exhibited (i) no more substrate inhibition and (ii) no more activation by Ins(1,3,4,5)P₄, but instead a competitive inhibition by this product. This inhibition by Ins(1,3,4,5)P₄ instead of an activation can directly be seen from the data in Fig. 4B, where again (see above) initial enzyme activities at 1 μM Ins(1,4,5)P₃ were measured in presence of increasing initial concentrations of Ins(1,3,4,5)P₄. Furthermore, this truncated enzyme exhibited absolutely normal Michaelis-Menten type substrate kinetics with respect to Ins(1,4,5)P₃ with and without Ins(1,3,4,5)P₄ present (complete

### FIG. 1.

**Domain structure of RnIP3K-C.** The RnIP3K-C possesses essentially the same domain structure as the hitherto known isoforms from rat and human. From N to C terminus the N-terminal domain, the PEST-motif (PEST), the calmodulin binding domain (CaM), and the catalytic domain are shown as boxes. The amino acid sequence of the rat C isoform is compared with the sequences of the rat A and B isoforms and the human A, B, and C isoforms (see Ref. 9 and citations therein) (GenBank™ accession no. AJ242781), and the percentage of identity is shown.

| Domain          | Rat C | Rat A | Rat B | Hs A | Hs B | Hs C |
|-----------------|-------|-------|-------|------|------|------|
| N-terminal      | 52    | 33    | 30    | 37   | 30   | 54   |
| PEST            | 39    | 57    | 57    | 59   | 59   | 97   |
| CaM             | 65    | 65    | 65    | 65   | 65   | 65   |

### FIG. 2.

**Expression pattern of RnIP3K-C mRNA in rat tissues.** A single transcript of 3.4 kb was present predominantly in heart, brain, and testes; at lower levels in lung, liver, and kidney; and almost undetectably in spleen and skeletal muscle (Fig 2A). The expression pattern of RnIP3K-C mRNA was confirmed by semiquantitative RT-PCR with a set of cDNAs prepared separately (Fig. 2C). The RT-PCR data correlate exactly with the results of both Northern blot experiments.
substrate kinetic data not shown). Its true \( K_m \) value for Ins(1,4,5)P$_3$ derived from linear extrapolations of \( K_{\text{m(app)}} \) values determined at differing inhibiting Ins(1,3,4,5)P$_4$ concentrations (see “Experimental Procedures” and data not shown) was 11-fold increased to 2.2 (± 0.3) \( \mu \text{M} \) as compared with the enzyme containing the CaM binding domain (0.21 (± 0.06) \( \mu \text{M} \); see Fig. 3A) but uncomplexed with Ca$^{2+}$-CaM. This \( K_{\text{m(app)}} \) versus Ins(1,3,4,5)P$_4$ replot resulted in a negative x intercept equaling \( -K_{\text{m(app)}} \) of 4.3 (± 0.6) \( \mu \text{M} \), which compares well with the apparent \( K_p \) value of 4.18 \( \mu \text{M} \) derived for inhibition at 1 \( \mu \text{M} \) Ins(1,4,5)P$_3$ (Fig. 4B). Apparently the presence of the CaM binding domain is not only directly responsible for the observed product activation but also for the strong substrate inhibition of the Ca$^{2+}$-CaM free enzyme and an increased substrate affinity (see above). The obvious isosteric interaction between substrate and product at the substrate/product binding site of the catalytic domain devoid of the CaM binding domain is characterized by markedly lower apparent binding affinities for InsP$_4$ (\( K_{\text{I(InsP4)}} \) = 4.3 \( \mu \text{M} \); see also the inhibition at 1 \( \mu \text{M} \) InsP$_4$; Fig. 4B) and for Ins(1,4,5)P$_3$ (\( K_{\text{I(InsP3)}} \) = 2.2 \( \mu \text{M} \)) than the ones derived from product activation (\( K_{\text{I(app)}} \) = 0.52 \( \mu \text{M} \); see Fig. 4A) and substrate inhibition (\( K_I = 0.09 \) (± 0.01) \( \mu \text{M} \); see Fig. 3A) in the enzyme containing the CaM binding domain. The abolishment of Ins(1,3,4,5)P$_4$-dependent product activation as well as the relief of the strong substrate inhibition by CaM binding to the enzyme are thus most likely a consequence of a direct competitive interaction between Ca$^{2+}$-CaM and Ins(1,3,4,5)P$_3$ and/or Ins(1,4,5)P$_3$ at the CaM binding domain. The latter domain of IP3K-C thus is likely to be itself an allosteric substrate and product binding domain with higher affinities for both ligands than the catalytic domain devoid of the CaM binding domain.

Unexpectedly, this isoform is also able to phosphorylate a second biological InsP$_3$ isomer, namely Ins(2,4,5)P$_3$, with relatively high efficiency. In assays where we used Ins(2,4,5)P$_3$ instead of Ins(1,4,5)P$_3$ as a substrate, we were able to detect a low but consistent enzyme activity of ≈50 milliunits/mg (Fig. 5A). The maximal activity was twice as high in the presence of Ca$^{2+}$-CaM as in the absence, but also the \( K_{\text{m(app)}} \) increased from 1.5 to ≈5 \( \mu \text{M} \) under these conditions (Fig. 5B). Although these maximal activities are ≈50 and 100-fold lower, respectively, than the corresponding \( V_{\text{max(app)}} \) values for Ins(1,4,5)P$_3$ (see above), they are in the range of activities reported for the conversion of Ins(1,3,4)P$_3$ to Ins(1,3,4,6)P$_4$ and Ins(1,3,4,5)P$_4$ by purified Ins(1,3,4)P$_4$ 5/6-kinase (46). To identify the product of this reaction, MDD-HPLC analysis of the reaction mixture after 0, 1, and 2 h of incubation with the enzyme was performed. The comparison of the inositol phosphates present in these reaction mixtures against a mixture of standard inositol phosphate isomers revealed a predominating reaction product co-eluting with Ins(2,4,5,6)P$_4$ (Fig. 6). Because both the substrate and the product appear to be >95% pure, this clearly indicates the phosphorylation of Ins(2,4,5)P$_3$ exclusively to Ins(2,4,5,6)P$_4$. This Ins(2,4,5)P$_3$ 6-kinase thus is an authentic side activity of RnIP3K-C. Small peaks of Ins(1,3,4,5)P$_4$ and Ins(1,3,4,6)P$_4$ also detected by MDD-HPLC analysis after incubation of Ins(2,4,5)P$_3$ with RnIP3K-C can be explained (i) by a small contamination of Ins(2,4,5)P$_3$ with Ins(1,4,5)P$_3$ (being converted to Ins(1,3,4,5)P$_4$), as well as (ii) by a further contamination of Ins(2,4,5)P$_3$ with a small amount of Ins(3,4,5)P$_3$, the latter one apparently also phosphorylated, like Ins(2,4,5,6)P$_4$ at the 6-hydroxy group to Ins(3,4,5,6)P$_4$.

To find out whether the ability of IP3K-C to convert Ins(2,4,5)P$_3$ to Ins(2,4,5,6)P$_4$ is an exclusive property of this isoform of IP3K, we expressed catalytic forms of rat IP3K-B and avian IP3K-A, both also containing the catalytic and the adjacent CaM binding domain but no N-terminal domain (data not shown). In both cases we found the same type of specific phosphorylation of this substrate at the hydroxyl group 6. In IP3K-A, which is only expressed in neurons, testes, and avian red blood cells (9, 27), a high \( V_{\text{max}} \) of 382 milliunits/mg and a \( K_m \) of 3.9 \( \mu \text{M} \) were derived in absence of CaM and similar values also in presence of 0.1 \( \mu \text{M} \) Ca$^{2+}$-CaM. The more ubiquitously expressed IP3K-B (28) revealed \( V_{\text{max}} \) and \( K_m \) values of 11.5 milliunits/mg and 1.3 \( \mu \text{M} \), respectively, in absence of CaM and of 24.5 milliunits/mg and 4.6 \( \mu \text{M} \), respectively, in presence of 0.1 \( \mu \text{M} \) Ca$^{2+}$-CaM (kinetics not shown). Among the two more ubiqui-

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Fig. 2. Tissue distribution of RnIP3K-C. A, Northern blot analysis of 2 \( \mu \text{g} \) of poly(A)$^+$ RNA from various rat tissues hybridized with a full-length $^{32}$P-labeled RnIP3K-C probe showed a single band of 3.4 kb in all tissues examined; by far the lowest amounts were present in spleen and skeletal muscle. B, Northern blot analysis of 15 \( \mu \text{g} \) of total RNA from selected rat tissues. A single transcript of 3.4 kb can be detected in taste and tongue epithelium, whereas only faint signals are present in brain and kidney because of the use of total RNA in this experiment. The blot was stripped and hybridized with an actin probe by the Northern experiment in B was confirmed by semiquantitative RT-PCR. In accordance with both Northern blots, the signals for brain and kidney are weaker whereas again skeletal muscle contains the lowest amount of RnIP3K-C mRNA. Hypoxanthine-guanine-phosphoribosyltransferase amplification served as control for RT-PCR and gel analysis (lower panel of C). Details of the experimental procedures are described under “Experimental Procedures.”

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**Fig. 3.** Enzymatic properties of RnIP3K-C: substrate inhibition by Ins(1,4,5)P$_3$ and activation by Ca$^{2+}$-CaM. Enzyme-coupled optical assays based on NADH consumption and conditions are described under “Experimental Procedures.” Enzyme activities of CBD-RnIP3K-C in presence of various initial concentrations of Ins(1,4,5)P$_3$ were measured in the absence (panel A) and presence (panel B) of 0.1 μM Ca$^{2+}$-CaM. In panel A data from one single transient starting at 4 μM Ins(1,4,5)P$_3$ (chevrons without error bars) were combined. Boxes contain parameters, and lines represent data fits for two models of substrate inhibition. Four-parameter box and unbroken line, general non-competitive substrate inhibition: $V = V_{max}^A[S]/K_m + [S] = I_{max}^A[S]/K_t + [S]$; four-parameter box and broken line, ordered Bi-Bi reaction with dead end $E_A$ complex (described on pp. 822–825 in Ref. 47); $V = V_{max}^A[S]/K_{m[A]} + V_{max}^B[S]/K_{m[B]} + [A]*1 + K_{m[BP]}(1 + [A]/K_t); K_{m[A]} = K_{m[BP]}/[B]; A = Ins(1,4,5)P_3 = first substrate bound; B = ATP = second substrate bound; $E_A$ formed by binding of a second A before binding of B; restraints: $K_{m[A]} = 35$ μM = constant. In panel A the value $K_{m[A]} (= K_t$ for A) derived from the data in panel B was employed to obtain convergence of the iterative fit. The activation by Ca$^{2+}$-CaM (panel C) was analyzed by initial activity assays at 5 μM initial Ins(1,4,5)P$_3$ concentration, respectively. For an estimation of the $K_t$ value for binding of the activator (assumed to be identical to $K_t$ values for activation), a simple hyperbolic association model ($V = B_0 + (B_{max} - B_0)*[A]/(K_t + [A])$) was employed; $B_0 = V_{no bound activator}, B_{max} = V_{max bound activator}$. Derived parameters are given in the boxes, and the fitted functions are plotted together with data points.

uitously expressed IP3K isoforms, IP3K-B and IP3K-C, the latter exhibits ~4.5-fold higher specific activity toward Ins(2,4,5)P$_3$ in absence and in presence of Ca$^{2+}$-CaM, whereas the $K_m$ values for Ins(2,4,5)P$_3$ are not significantly different between these two isoforms. We also tested whether the initial presence of activator Ins(1,3,4,5)P$_4$ together with Ins(2,4,5)P$_3$ or its addition during the reaction could further increase the specific activity of IP3K-C for this alternative substrate but no significant effect, either activation or inhibition, was observed (data not shown). However, in the other two isoforms, A and B, Ins(1,3,4,5)P$_4$ exhibited a strong competitive inhibition of conversion of Ins(2,4,5)P$_3$. IP3K-C thus is the isoform better suited to perform this reaction in tissues and cell types expressing both isoform B and C because of its 5-fold higher $V_{max}$ against this substrate and the absence of competitive inhibition by Ins(1,3,4,5)P$_4$. We determined in different tissues and cell types by direct mass analysis of inositol phosphates using MDD-HPLC whether Ins(2,4,5,6)P$_4$ is resent and is increased after phospholipase C stimulation and could confirm both phenomena (data not shown).

We performed several optical assays to confirm the reversibility and the degree of Ca$^{2+}$-CaM activation of the enzyme. One of these experiments is shown in Fig. 7. The reactions were started at high Ins(1,4,5)P$_3$ in the presence of 20 μM free Ca$^{2+}$, but in absence of CaM. After 1–2 min, when the enzyme exhibited basal substrate inhibited activity (see above), 0.1 μM CaM was added and the reaction followed. Thus, both the degree of CaM activation (7-fold in the example shown in Fig. 7) and the product activation and decrease of substrate inhibition could be directly demonstrated (see the activity derivations plotted together with the optically assayed decrease of NADH, which was converted into an equimolar decrease of Ins(1,4,5)P$_3$ in Fig. 7). The addition of 125 μM EGTA apparently led to the dissociation of CaM and the observed re-decrease of activity. Finally, by
were transiently expressed in NRK 52E cells, and the localization of the fusion proteins was determined by inspection using fluorescence microscopy. (Typical images of the different types of localization are shown in Fig. 8A.) Both arrangements of protein sequences in the fusion protein were used to examine steric effects of the fused fluorescent protein influencing the localization of the fusion protein. In most of the transfected cells, the fusion proteins were detected evenly distributed between nucleus and cytoplasm or exclusively in the cytoplasm. In a small minority of cells, they were localized predominantly in the nucleus (Fig. 8B; N-tagfull C-tagfull in Table I). This localization pattern was not an artifact of EGFP tagging, because the arrangement of protein sequences had no obvious impact on the localization pattern (N-tagfull versus C-tagfull in Table I). In control experiments where we expressed EGFP alone, all transfected NRK 52E cells showed the same localization, namely an even distribution between nucleus and cytoplasm (data not shown), ruling out a targeting activity of EGFP itself. Small proteins can enter the nucleus of a cell by passive diffusion, but proteins larger than ~40 kDa require an NLS for active translocation through the nuclear pore complex (11). The molecular weight of the fusion protein (101.4 kDa) clearly exceeds this limit. This obviously active translocation of IP3K-C into the nucleus seems to be in contradiction to the exclusively cytoplasmic localization described by Dewaste et al. (10). Therefore, we examined the possibility of an additional nuclear export activity by incubation of NRK 52E cells expressing the fusion protein N-tagfull with the export inhibitor LMB. After 6 h of LMB treatment, no cells with an exclusively cytoplasmic localization of the fusion protein were observed (Fig. 8B; N-tagfull/no LMB versus N-tagfull/+LMB in Table I). The increase of cells showing an even distribution and the decrease of cells showing an exclusively cytoplasmic localization of the fusion protein are extremely significant (p < 0.001). Furthermore, the proportion of cells with a predominantly nuclear localization seems to be increased (0.05 < p < 0.1). Therefore, the fusion protein is obviously transported out of the nucleus by an active, LMB-sensitive mechanism. Similar results were also obtained by additional transfection experiments using PC12 cells (data not shown). In summing up, the RnIP3K-C seems to be a nucleocytoplasmic shuttling protein with both nuclear import and nuclear export activity.

The N-terminal Domain Determines the Nucleocytoplasmic Shuttling of RnIP3K-C—Our results (see above) indicate that RnIP3K-C is a nucleocytoplasmic shuttling protein, but the positions of potential sites possessing nuclear import and export activity, respectively, are unknown. The N-terminal domains of the three IP3K isoforms are highly diverse and mainly uncharacterized in their function(s), whereas the other parts of the enzymes are conserved and well studied (Fig. 1). Therefore, these domains are preferred candidates to determine the intracellular targeting of the different isoforms. Indeed, the IP3K isoform A is localized to F-actin and dendritic spines by its N terminus (24). To narrow down sites exhibiting nuclear import or export activity, we fused the N-terminal domain of RnIP3K-C (aa 1–379) with an N-terminal EGFP tag (N-tagNterm) and a C-terminal EGFP tag (C-tagNterm), respectively. The localization pattern of both EGFP-tagged fragments is comparable with that of the full-length fusion proteins (N-tagNterm versus N-tagfull, C-tagNterm versus C-tagfull in Table I), as determined by fluorescence microscopy of transiently transfected NRK 52E cells. An effect of EGFP tagging can be ruled out, because the pattern is obviously not influenced by the arrangement of protein sequences in the fusion protein (N-tagNterm versus C-tagNterm in Table I). The nuclear entry of the EGFP-tagged fragments still requires an active import, be-
cause their molecular size (67.2 kDa) rules out a passive diffusion into the nucleus. Thus, the N-terminal domain of RnIP3K-C tagged with EGFP seems to possess a nuclear import activity comparable with that of the full-length fusion protein, although no classical NLS (25) was revealed by consensus sequence search (data not shown). To further examine potential targeting mechanisms, NRK 52E cells expressing an EGFP-tagged fragment (N-tagNterm) were incubated with LMB. After an LMB incubation of 6 h, no cells with an exclusively cytoplasmic localization were observed (N-tagNterm/no LMB versus N-tagNterm/+LMB in Table 1). Both the decrease of the cell number with an exclusively cytoplasmic localization and the increase of cells with an even distribution of the fragment are extremely significant ($p < 0.001$), whereas the proportion of cells showing a predominantly nuclear localization seems to be increased ($0.05 < p < 0.10$). Therefore, the EGFP-tagged N-terminal domain of RnIP3K-C seems to act as a nuclear import signal.

**Fig. 5.** Phosphorylation of Ins(2,4,5)P$_3$ by RnIP3K-C. An enzyme-coupled optical assay as described under “Experimental Procedures” was used to determine the enzymatic parameters $K_m$ and $V_{max}$ of CBD-RnIP3K-C for the substrate Ins(2,4,5)P$_3$. They were determined in the absence (panel A) and presence (panel B) of 0.1 $\mu$M Ca$^{2+}$-CaM, respectively. Other conditions are as given in Fig. 3.

**Fig. 6.** MDD-HPLC analysis of the phosphorylation products of Ins (2,4,5)P$_3$ formed by RnIP3K-C. Recombinant enzyme (CBD-RnIP3K-C) was incubated with Ins(2,4,5)P$_3$ under the standard optical assay conditions, and products were analyzed after 0, 1, and 2 h of incubation by MDD-HPLC as described under “Experimental Procedures.” The conversion of Ins(2,4,5)P$_3$ was nearly complete after 2 h of incubation. A standard mixture of inositol phosphates generated by limited acid hydrolysis of InsP$_6$ was separated with the same gradient (upper chromatogram) and used as an isomeric standard mixture. The same type of analysis was also performed with the reaction product(s) of Ins(1,4,5)P$_3$, and the only isomer formed was Ins(1,3,4,5)P$_4$ (chromatograms not shown).
terminal domain of RnIP3K-C demonstrates an LMB-sensitive nuclear export activity comparable with that of the full length fusion protein. To further narrow down potential sites possessing nuclear export activity, we analyzed the sequence of the N-terminal domain of RnIP3K-C. One candidate sequence (aa 318–326) precisely fits the NES consensus (Fig. 9) (11, 12, 26) and is completely conserved between human and rat protein (data not shown). This putative NES was deleted in an EGFP-tagged full-length fusion gene to further investigate its role in the intracellular targeting of IP3K-C. The NES deletion mutant was transiently expressed in NRK 52E cells, and its localization was examined by fluorescence microscopy. Now almost all cells showed an even distribution of the fusion protein between nucleus and cytoplasm comparable with LMB-treated cells expressing EGFP-tagged full-length proteins (C-tagfull, H9004 NESin Fig. 8B and Table I). The deletion of an internal sequence is a drastic operation, which can lead to misfolding and thus changes in the three-dimensional structure of a pro-
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| Table I  | Intracellular distribution of different EGFP/IP3K-C fusion proteins in NRK 52E cells and effect of LMB treatment |
|----------|---------------------------------------------------------------------------------|
|          | Intra cellular distribution<sup>a</sup>                                                                 |
|          | N                         | NC                        | C                          |
|----------|---------------------------|---------------------------|---------------------------|
| N-tag<sub>full</sub> |                           |                           |                            |
| No LMB       | 1.5 ± 1.9                 | [55.5 ± 4.4]*             | [42.9 ± 3.5]*              |
| + LMB         | 8.2 ± 4.8                 | 91.4 ± 4.3                | 0.3 ± 0.6                  |
| C-tag<sub>full</sub> | 1.8                      | 60.9                      | 37.3                      |
| C-tag<sub>full, ANES</sub> | 7.1 ± 3.1              | 93.0 ± 3.1                | 0.0 ± 0.0                  |
| C-tag<sub>full, mNES</sub> | 9.1 ± 4.6               | 90.4 ± 4.6                | 0.0 ± 0.0                  |
| N-tag<sub>full</sub> |                           |                           |                            |
| No LMB       | 0.9 ± 0.9                 | 61.7 ± 5.3*               | [37.4 ± 5.4]*              |
| + LMB         | 6.0 ± 4.0                 | 93.7 ± 3.5                | 0.3 ± 0.5                  |
| C-tag<sub>N term</sub> | 0.9                     | 73.2                      | 25.9                      |

<sup>a</sup> The proportion of transiently transfected NRK 52E cells showing predominantly nuclear (N), nuclear/cytoplasmic (NC), or cytoplasmic (C) localization of the fusion protein is indicated as the mean ± S.D. of three independent experiments. Typical images of the different types of localization are shown in Fig. 7A. A minimum of 100 undamaged cells per experiment were examined by inspection. Asterisks indicate an extremely significant (<i>p</i> < 0.001) difference between untreated and LMB-treated cells in the percentage of cells showing the examined localization. The structure of the different fusion proteins is further described in the text.

<sup>b</sup> This experiment was performed only once.

Discussion

IP3K-C Is Specialized for Continuous Basal Ins(1,4,5)P<sub>3</sub> Phosphorylation in Unstimulated Cells—Comparison of the RnIP3K-C amino acid sequence to the other known vertebrate IP3K sequences yields a higher degree of identity to its human orthologue than to the other two known rat isoforms (our results). A result that is also true if A and B isoform sequences are compared within and between species (data not shown). Therefore, it seems plausible that the three isoforms diverged early in vertebrate evolution to fulfill different tasks within the inositol phosphate metabolism. Since then, considerable selective pressure to conserve these specific functions of each isoform must have kept the sequence of the individual isoforms relatively unchanged during successive speciation events. One possibility for the formation of isoforms is their adaptation to cell- or tissue-specific expression. This type of specialization has obviously been adopted by the A isoform, which shows a unique expression in neurons and in testis (27). On the other hand, the available evidence for the B (28) and, with some restrictions, C isoform (10) instead suggests a relatively broad distribution of both isoforms across tissues and cell types, which directs toward other kinds of functional specialization of these two isoforms. Still, the hitherto known tissue distribution of the C isoform indicates variation between species. Our results obtained with rat tissues show expression of the C isoform in heart, brain, lung, liver, kidney, and testis and most abundantly in an epidermal tissue, namely tongue and taste bud epithelium. Almost no RnIP3K-C mRNA was found in skeletal muscle. In contrast, Dewaste et al. (10) detected the human C isoform mRNA in skeletal muscle but not in brain and kidney. Assuming that A, B, and C isoforms have at least overlapping expression patterns, a specialization of these isoforms could pertain either to their intracellular localization or to their catalytic properties. In these respects our study has highlighted considerable differences between A and B isoforms on the one hand and the C isoform on the other hand. Although A and B isoforms are markedly inhibited by the product of the IP3K reaction Ins(1,3,4,5)P<sub>4</sub> (our own results for IP3K-B; data not shown); see Ref. 29 for IP3K-A), this enzyme product is in fact an allosteric activator of the C isoform in the absence of Ca<sup>2+</sup>-CaM (our results). Because the whole N-terminal domain upstream of the CaM binding domain was missing in the enzyme form employed for the kinetic analyses, the allosteric site where Ins(1,3,4,5)P<sub>4</sub> binds can only reside in the catalytic domain or in the CaM binding domain. The strong degree of sequence identity between all three isoforms in their catalytic domain does not make it likely that such an allosteric site is residing in a segment of the catalytic domain. Rather, the fact that this product activation by Ins(1,3,4,5)P<sub>4</sub> is completely abolished by addition of Ca<sup>2+</sup>-CaM (our results) or by deletion of the CaM binding domain (our results) directs toward a localization of this allosteric site in this domain. An inspection of the CaM binding domains of all three isoforms reveals that the C isoform contains an additional basic residue at position Lys<sup>281</sup>, which might be responsible for a specific interaction of the uncomplexed CaM binding domain with both the active site and the two inositol phosphates Ins(1,4,5)P<sub>3</sub> and Ins(1,3,4,5)P<sub>4</sub> in a way inhibiting or activating substrate conversion, respectively. Because the allosteric activation by Ins(1,3,4,5)P<sub>4</sub> is half-maximal at a concentration that this product easily reaches intracellularly (0.2–20 μM depending on cell type and stimulation of corresponding cells; our own data not shown), this activation is likely to represent a physiological “feed-back” activation mechanism being active only under resting Ca<sup>2+</sup> concentrations, i.e. in cells exhibiting a slow release of InsP<sub>3</sub> insufficient to release Ca<sup>2+</sup>. The low K<sub>m</sub>/high affinity for Ins(1,4,5)P<sub>3</sub> would further enhance this basal Ins(1,4,5)P<sub>3</sub> conversion. On the other hand, the unusual phenomenon of substrate inhibition that is seen with the Ca<sup>2+</sup>-CaM free enzyme at higher Ins(1,4,5)P<sub>3</sub> concentrations in vitro and is also likely to be mediated by the CaM binding domain (see “Results”) does not imply that the enzyme is switched off physiologically at a higher InsP<sub>3</sub> level. Namely, this normally induces a cellular...
Ca\(^{2+}\) release with concomitant Ca\(^{2+}\)-CaM activation of the enzyme; thus, substrate inhibition is strongly diminished (Fig. 3, compare B and A). In other words, the degree of activation by Ca\(^{2+}\)-CaM, which is markedly higher for the rat C isofrom than that reported for the human one (10), increases with higher Ins(1,4,5)P\(_{3}\) (our results). These specific enzymatic features imply a completely different mode of function for the C isofrom in living cells. Although A and B isoforms are boosted in their activity as a consequence of a signal transduction event liberating Ins(1,4,5)P\(_{3}\) from the cellular plasma membrane, as long as there is no significant accumulation of Ins(1,3,4,5)P\(_{4}\), the C isofrom rather seems suited to function during the intervals between signaling events, when there is only little Ins(1,4,5)P\(_{3}\) available in the cytosol and no Ca\(^{2+}\)-CaM present, and, in contrast to isoforms A and B, it keeps its high affinity for Ins(1,4,5)P\(_{3}\) even when Ins(1,3,4,5)P\(_{4}\) has been already formed or even strongly accumulated. IP3K-C thus could keep the “resting” Ins(1,4,5)P\(_{3}\) very low and provide a basal Ins(1,3,4,5)P\(_{4}\) production in the absence of stimulatory signals from the exterior. Because Ins(1,4,5)P\(_{3}\) is stored, the enzyme might “channel” most of the basal Ins(1,4,5)P\(_{3}\) flux into the “anabolic” route. One could argue that a permanent intracellular generation of Ins(1,3,4,5)P\(_{4}\) might be a necessary event to maintain stimulus independent, constitutive cell growth and differentiation. Such an assumption is substantiated by the fact that Ins(1,3,4,5)P\(_{4}\) is thought to be the first metabolite on an “anabolic pathway” leading to the formation of highly phosphorylated inositol phosphates. Some of these have been implicated in protein phosphorylation, mRNA export, and thus normal cell growth in yeast, DNA-dependent protein kinase activation, and thus normal DNA recombination and DNA repair during the propagation of the cell cycle, and regulation of apoptosis (see Ref. 30 and citations therein; see also Refs. 31 and 32).

**IP3K-C Can Phosphorylate Ins(2,4,5)P\(_{3}\) at Its 6-Hydroxy Group**—Another specific feature of the C isofrom is its ability to bind and convert the alternative substrate Ins(2,4,5)P\(_{3}\) to Ins(2,4,5,6)P\(_{4}\). A synergistic control of Ca\(^{2+}\) mobilization from intracellular stores in mouse lymphoma cells by Ins(2,4,5,6)P\(_{4}\) and Ins(1,3,4,5)P\(_{4}\) has been reported (33). This isomer is possibly produced by enzymatic conversion of a cyclic inositol phosphate intermediate (cIns(1,2,4,5)P\(_{4}\)) by Ca\(^{2+}\)-activated phospholipase C-catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate (44, 45). In addition to the removal of once formed Ins(2,4,5)P\(_{3}\), this activity of rat IP3K-C may constitute an alternative route in the metabolism of higher inositol phosphates. Until now there has been no success in isolation and characterization of any enzyme activity that would perform the conversion of Ins(2,4,5)P\(_{3}\) to an InsP\(_{4}\) isomer in animal cells. This novel InsP\(_{4}\) isomer generated by IP3K-C may be crucial for the formation of InsP\(_{6}\), as in rat brain the main activity producing InsP\(_{6}\) from an InsP\(_{5}\) precursor relies on the Ins(1,2,4,5,6)P\(_{5}\) isomer (34) and because the specific activity of the recently discovered human Ins(1,3,4,5,6)P\(_{5}\)-2-kinase is quite low and its expression in some tissues is apparently poor (37), although in each of these tissues InsP\(_{6}\) is present. Because of the 7-fold lower apparent affinity of IP3K-C for Ins(2,4,5)P\(_{3}\) than for Ins(1,4,5)P\(_{3}\), this enzyme will first convert all Ins(1,4,5)P\(_{3}\) to Ins(1,3,4,5)P\(_{4}\) in a cell with low Ca\(^{2+}\); and then, unfluenced by the presence of this product, it will efficiently convert Ins(2,4,5)P\(_{3}\) to Ins(2,4,5,6)P\(_{4}\). IP3K-A and IP3K-B will not be able to convert Ins(2,4,5)P\(_{3}\) as well although exhibiting activity toward this substrate (our results), because in these isoforms the strong competitive inhibition by Ins(1,3,4,5)P\(_{4}\) once formed will prevent binding of this alternative substrate. In a number of tissues and cell lines, the presence of Ins(2,4,5,6)P\(_{4}\) could be confirmed; in OKT-3-stimulated Jurkat cells, a delayed formation of Ins(2,4,5,6)P\(_{4}\) after T cell receptor stimulation could be demonstrated by sensitive micro-MDD-HPLC analysis (data not shown). In these cells there is always a significant amount of Ins(1,2,4,5,6)P\(_{5}\) and/or its enantiomer Ins(2,3,4,5,6)P\(_{5}\) present, making it likely that there is a metabolic interrelationship between Ins(2,4,5,6)P\(_{4}\) formed by IP3K-C and one or both of these InsP\(_{5}\) isomers known to be convertible to InsP\(_{6}\). The low concentrations of Ins(2,4,5,6)P\(_{4}\) detected in cells and tissues imply that such enzyme converting Ins(2,4,5,6)P\(_{4}\) to InsP\(_{6}\) should have a high affinity/low \(K_m\) for Ins(2,4,5,6)P\(_{4}\).

**IP3K-C Actively Shuttles between Cytoplasm and Nucleus as a Result of a Nuclear Import Activity and a Nuclear Export Activity in Its N-Terminal Domain**—As shown in this study, RnIP3K-C is not only localized in the cytoplasm, but can also be targeted to the nucleus of the cell. A phospholipase C-dependent inositol phosphatase kinase pathway leading up to InsP\(_{6}\) in the nuclei of yeast cells has been described previously (35). Recent findings indicate that, in mammalian cells as well, inositol phosphate kinases in the nucleus act together with other enzymes of inositol phosphate metabolism (e.g. nuclear phosphatase C (Ref. 36)), forming a nuclear inositol phosphate signaling and phosphorylation pathway. The nuclear conversion of Ins(1,4,5)P\(_{3}\) to Ins(1,3,4,5)P\(_{4}\) can apparently be performed by inositol phosphate multikinase (22) and IP3K-C (our results), but no enzyme with nuclear localization additional to the inositol phosphate multikinase (22) is known to convert Ins(1,3,4,5)P\(_{4}\) to Ins(1,3,4,5,6)P\(_{5}\). This nuclear pathway may end up with highly phosphorylated inositol phosphates (e.g. InsP\(_{5}\) (Ref. 37)), part of which are pyrophosphorylated (38, 39) by nuclear inositol hexakisphosphate kinase isofrom 2 (40).

In this study, we have shown that a full-length RnIP3K-C fusion protein with EGFP readily enters the nuclei of mammalian cells and treatment with LMB or inactivation of a potential NES by deletion or point mutation promotes its nuclear accumulation. The size of the fusion protein rules out a nucleocytoplasmic shuttling by simple diffusion through the nuclear pore complexes (11). Therefore, active nucleocytoplasmic translocation mechanisms must exist. Our results suggest that the intracellular distribution of RnIP3K-C is regulated by two mechanisms, an uncharacterized active nuclear import mechanism additional to an LMB-sensitive nuclear export mechanism. The nuclear import could be either mediated by a non-classical NLS (41) or by a co-transport mechanism (42), because no classical NLS was identified.

Intriguingly, both localization-determining activities are localized in the N-terminal domain of the protein (our results), and the association of IP3K-A with F-actin and dendritic spines is also mediated by a targeting domain in its N terminus (24). Therefore, it will be interesting to determine whether IP3K-B (28) is also targeted to specific intracellular regions (43) by its N-terminal domain. Furthermore, the targeting of IP3K-C may be cell type-dependent, because the intracellular localization observed in NRK 52E cells (our results) seems to differ from the distribution reported by Dewaste et al., using transfected COS-7 cells. In their study a nearly complete nuclear exclusion of the enzyme was revealed by activity determinations and Western blot analysis (10). Therefore, future experiments should focus on the identification of factors that influence the relative strength of nuclear import and nuclear export activities of IP3K-C in different cell types.

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Rat Inositol 1,4,5-Trisphosphate 3-Kinase C Is Enzymatically Specialized for Basal Cellular Inositol Trisphosphate Phosphorylation and Shuttles Actively between Nucleus and Cytoplasm

Marcus M. Nalaskowski, Uwe Bertsch, Werner Fanick, Malte C. Stockebrand, Hartwig Schmale and Georg W. Mayr

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