A Novel Receptor-mediated Regulation Mechanism of Type I Inositol Polyphosphate 5-Phosphatase by Calcium/Calmodulin-dependent Protein Kinase II Phosphorylation

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Inhibition of Type I 5-Phosphatase by Phosphorylation

The enzyme has been immunoprecipitated from human astrocytic 1321N1 cells homogenates and separated by SDS/PAGE, as described under "Experimental Procedures." The Coomassie Blue-stained 43-kDa gel band was excised (see Fig. 1, lane 1) and then excised (circle, lane 1) for mass spectrometry analysis. The standard molecular masses (kDa) are indicated in the margin.

![Fig. 1. Immunodetection and immunoprecipitation of type I 5-phosphatase from astrocytic and basophilic mast cells by antihuman 43 kDa 5-phosphatase antibodies. A, a 5-μl aliquot of crude cell lysate (~25 μg of proteins) was separated by SDS/PAGE, and the 5-phosphatase (indicated by an arrow) was immunodetected in the presence of anti-43-kDa 5-phosphatase antibodies in rat brain cortical astrocytes (lane 1), 1321N1 cells (lane 2), and RBL-2H3 cells (lane 3). B, immunoprecipitated 5-phosphatase from 1321N1 cells (100 μl of crude cell lysate, i.e. ~700 μg of proteins, with 2 μl of immune serum) was separated by SDS/PAGE, and the protein was stained by colloidal Coomassie Blue (lanes 1 and 2) and then excised (circle, lane 1) for mass spectrometry analysis. The standard molecular masses (kDa) are indicated in the margin.

| Peptide mapping |
|-----------------|
| **Theoretically calculated mass** | **Residues** | **Theoretical sequence** |
| Da              | Da            |                      |
| 1074.49         | 1074.52       | 116–123              |
| 1477.69         | 1477.70       | 285–295              |
| 1497.70         | 1497.71       | 225–236              |
| 1605.77         | 1605.80       | 284–295              |
| 2040.03         | 2040.02       | 296–313              |
| 2241.12         | 2241.09       | 315–319              |
| 2262.13         | 2262.05       | 318–319              |

**TABLE I**

Identification of Ins(1,4,5)P₃/Ins(1,3,4,5)P₄ 5-phosphatase from astrocytic 1321N1 cells by mass spectrometry

The enzyme has been immunoprecipitated from human astrocytic 1321N1 cells homogenates and separated by SDS/PAGE, as described under "Experimental Procedures." The Coomassie Blue-stained 43-kDa gel band was excised (see Fig. 1, lane 1). The protein was denatured with urea and digested with trypsin in the presence of ¹⁸O-labeled H₂O. The tryptic peptide mixture was adsorbed onto porous R2 and was analysed on a MALDI/reflectron time of flight mass spectrometer.

**PSD-based sequence verification of NH₂-KLFDYFNQEVFR-COOH**

| Residue No. | Theoretically calculated y ions | Theoretical sequence | Experimentally measured y ions | Experimental sequence |
|-------------|--------------------------------|----------------------|-------------------------------|-----------------------|
| Da          | Da                            |                      |                               |                       |
| 1           | 1477.71                       | K                    | -                             | -                     |
| 2           | 1364.63                       | L                    | -                             | -                     |
| 3           | 1217.56                       | F                    | -                             | -                     |
| 4           | 1102.53                       | D                    | 1102.59                       |                       |
| 5           | 939.47                        | Y                    | -                             | -                     |
| 6           | 792.40                        | F                    | -                             | -                     |
| 7           | 678.36                        | N                    | -                             | -                     |
| 8           | 559.30                        | Q                    | 559.68                        | Q                     |
| 9           | 421.26                        | E                    | 421.13                        | E                     |
| 10          | 322.19                        | V                    | 321.23                        | V                     |
| 11          | 175.12                        | F                    | 175.29                        | F                     |
| 12          | 19.02                         | R                    | -                             | R                     |

* Tryptic peptides are single-charged, and therefore, masses include one proton. Experimental and theoretical masses are monoisotopic masses, respectively. Mass accuracy is typically 0.1% or better. Theoretical masses were calculated considering normal cysteine and methionine and trypptic cleavage after lysine and arginine but not before proline. These seven peptides cover 88 out of the 412 amino acids (i.e. 21%) from the human primary structure. The peptide that has been analyzed by PSD is indicated by an asterisk.

The peptide with an experimental monoisotopic mass of 1605.777 Da has been selected as a parent ion for fragmentation by PSD generating the typical C-terminal ¹⁸O-labeled y-ion series. In the PSD spectrum, the parent ion presented a mass of 1605.22 Da. Theoretical monoisotopic masses were calculated considering normal cysteine and methionine. The dashes mean not detected. Analysis of the raw PSD data by using the Mascot algorithm gave the type I 5-phosphatase primary structure as the top-scoring protein.
**Table II**

Effect of Ca\(^{2+}\)-raising agonists and okadaic acid on Ins(1,4,5)P\(_3\) 5-phosphatase activity in rat brain cortical astrocytes and RBL-2H3 cells

Enzyme activity was assayed at 10 \(\mu\)M Ins(1,4,5)P\(_3\). 100% enzyme activity corresponded to 0.15 \(\mu\)mol/min \cdot mg and 0.22 \(\mu\)mol/min \cdot mg in rat cortical astrocytes and RBL-2H3 cells, respectively. O.A., okadaic acid. Each value is the mean of duplicates \(\pm\) S.D. The results are from one representative experiment out of four.

| Incubation conditions | Rat brain cortical astrocytes | RBL-2H3 cells |
|-----------------------|-------------------------------|---------------|
| No agent              | 100 ± 9                       | 100 ± 12      |
| Carbachol, 0.1 \(\mu\)M (30 s) | 98 ± 4                      | 103 ± 4       |
| Carbachol, 1 \(\mu\)M     | 62 ± 5                       | 66 ± 13       |
| Carbachol, 5 \(\mu\)M     | 47 ± 10                      | 47 ± 8        |
| Carbachol, 10 \(\mu\)M    | 41 ± 6                       | 47 ± 6        |
| Carbachol, 100 \(\mu\)M   | 43 ± 5                       | 45 ± 3        |
| UTP, 0.1 \(\mu\)M (30 s)  | 105 ± 11                     |               |
| UTP, 1 \(\mu\)M          | 69 ± 9                       | 101 ± 14      |
| UTP, 10 \(\mu\)M         | 51 ± 12                      | 100 ± 14      |
| UTP, 100 \(\mu\)M        | 40 ± 12                      | 98 ± 11       |
| O.A. (100 nM; 30 min)    | 105 ± 11                     | 97 ± 13       |
| O.A. (100 nM; 30 min) + UTP, 10 \(\mu\)M, (30 s) | 104 ± 4       | 101 ± 14      |
| O.A. (100 nM; 30 min) + UTP, 10 \(\mu\)M, (30 s) | 74 ± 9        | 63 ± 8        |
| O.A. (100 nM; 30 min) + UTP, 10 \(\mu\)M, (30 s) | 22 ± 3        | 16 ± 6        |
| O.A. (100 nM; 30 min) + UTP, 10 \(\mu\)M, (30 s) | 59 ± 13       | 70 ± 8        |
| O.A. (100 nM; 30 min) + UTP, 10 \(\mu\)M, (30 s) | 116 ± 26      | 100 ± 14      |
| O.A. (100 nM; 30 min) + UTP, 10 \(\mu\)M, (30 s) | 101 ± 12      |               |

**Table III**

Effect of Ca\(^{2+}\)-raising agonists and okadaic acid on Ins(1,3,4,5)P\(_4\) 5-phosphatase activity in rat brain cortical astrocytes and RBL-2H3 cells

Enzyme activity was assayed at 5 \(\mu\)M Ins(1,3,4,5)P\(_4\). 100% enzyme activity corresponded to 20 nmol/min \cdot mg and 28 nmol/min \cdot mg in rat cortical astrocytes and RBL-2H3 cells, respectively. O.A., okadaic acid. Each value is the mean of duplicates \(\pm\) S.D. The results are from one representative experiment out of three.

| Incubation conditions | Ins(1,3,4,5)P\(_4\) 5-phosphatase activity |
|-----------------------|-------------------------------------------|
| No agent              | Rat brain cortical astrocytes | RBL-2H3 cells |
| Carbachol, 0.1 \(\mu\)M (30 sec) | 107 ± 8                       | 93 ± 11       |
| Carbachol, 1 \(\mu\)M     | 69 ± 9                       | 69 ± 12       |
| Carbachol, 10 \(\mu\)M    | 44 ± 9                       | 45 ± 11       |
| Carbachol, 100 \(\mu\)M   | 50 ± 5                       | 45 ± 9        |
| UTP, 0.1 \(\mu\)M (30 s)  | 100 ± 17                     |               |
| UTP, 1 \(\mu\)M          | 69 ± 12                      |               |
| UTP, 10 \(\mu\)M         | 48 ± 8                       |               |
| UTP, 100 \(\mu\)M        | 39 ± 6                       |               |
| O.A. (100 nM; 30 min)    | 94 ± 10                      |               |
| O.A. (100 nM; 30 min) + UTP (10 \(\mu\)M; 30 s) | 21 ± 5        |               |
| O.A. (100 nM; 30 min) + UTP (10 \(\mu\)M; 30 s) | 101 ± 10      | 107 ± 16      |
| O.A. (100 nM; 30 min) + UTP (10 \(\mu\)M; 30 s) | 104 ± 4        | 101 ± 14      |
| O.A. (100 nM; 30 min) + UTP (10 \(\mu\)M; 30 s) | 74 ± 9        | 63 ± 8        |
| O.A. (100 nM; 30 min) + UTP (10 \(\mu\)M; 30 s) | 52 ± 13       | 49 ± 13       |
| O.A. (100 nM; 30 min) + UTP (10 \(\mu\)M; 30 s) | 50 ± 15        | 45 ± 11       |
| O.A. (100 nM; 30 min) + UTP (10 \(\mu\)M; 30 s) | 50 ± 5        | 45 ± 9        |

Ca\(^{2+}\)-raising agents. This effect appears quite general; indeed, treatment of rat brain cortical astrocytes, human astrocytoma 1321N1 cell line, and rat basophilic leukemia RBL-2H3 cell line with carbachol provoked a transient increase in the phosphorylation of the 43-kDa isoform and a corresponding decrease in enzymic activity.

**Experimental Procedures**

*Generation of Antibodies Dressed against the N-terminal Part of Human 43-kDa Inositol Polyphosphate 5-Phosphatase*—An immune serum recognizing a 16-amino acid peptide (i.e. MALHCQEFGGKYYAS) corresponding to amino acids 49–64 of human 43-kDa 5-phosphatase (28) coupled to hemocyanin has been generated in rabbits. It was verified that the antibodies (dilution 1:1000) immunodetected and immunoprecipitated recombinant type I 5-phosphatase. An aliquot of 2 \(\mu\)l of crude immune serum was used for immunoprecipitation experiments. For competition studies, 5 \(\mu\)g of the corresponding peptide were added for 1 h to the diluted serum before immunodetection.

*Preparation of Rat Brain Cortical Astrocytes*—Primary cultures of rat cerebral cortex astrocytes were established using dissociated rat cerebral tissue at 2 or 3 days after birth, according to methods previously described (29). Briefly, dissected cortical tissue was washed and then dissociated by gentle repeated pipetting in modified Eagle’s medium containing 1 mM sodium pyruvate, 10% fetal calf serum, 2% penicillin/streptomycin, and 1% fungizone. Cells were detached by gravity for 5 min, and the supernatant was saved. Cells were diluted in supplemented cell medium and plated in 10-cm-diameter or 22 × 22-cm-square dishes at 37 °C with 5% CO\(_2\). When astrocytes were adherent, dishes were agitated overnight, and the medium was changed.
TABLE IV
Effect of TPA, calphostin C, and forskolin on Ins(1,4,5)P3 5-phosphatase activity in rat brain cortical astrocytes and RBL-2H3 cells

| Incubation conditions | Rat brain cortical astrocytes | RBL-2H3 cells |
|-----------------------|-------------------------------|---------------|
| No agent              | 100 ± 11                      | 100 ± 7       |
| Carbachol (10 μM; 30 s) | 40 ± 9                        | 44 ± 10       |
| TPA, 0 s (500 nM)     | 99 ± 5                        | 99 ± 13       |
| TPA, 15 s             | 101 ± 13                      | 99 ± 13       |
| TPA, 1 min            | 106 ± 20                      | 103 ± 9       |
| TPA, 5 min            | 92 ± 8                        | 97 ± 11       |
| Calphostin C (300 nM; 30 min) + carbachol (10 μM; 30 s) | 43 ± 23          | 45 ± 9       |
| Calphostin C (300 nM; 30 min) + UTP (10 μM; 30 s) | 42 ± 11          |               |
| Forskolin, 30 s (10 μM) | 103 ± 8                      | 99 ± 13       |
| Forskolin, 1 min      | 100 ± 11                      | 103 ± 12      |
| Forskolin, 5 min      | 89 ± 9                        | 97 ± 16       |
| Forskolin, 10 min     | 103 ± 5                       | 101 ± 6       |

TABLE V
Effect of the two Ca2+/CaM kinase II inhibitors KN-93 and KN-62 as well as the noneffective structural analogs KN-92 and KN-04 on Ins(1,4,5)P3 5-phosphatase inhibition in stimulated rat brain cortical astrocytes and RBL-2H3 cells

| Incubation conditions | Rat brain cortical astrocytes | RBL-2H3 cells |
|-----------------------|-------------------------------|---------------|
| No agent              | 100 ± 19                      | 100 ± 6       |
| KN-93, 0 μM (30 min) + carbachol (10 μM; 30 s) | 36 ± 8                        | 40 ± 9       |
| KN-93, 0.1 μM (30 min) + carbachol (10 μM; 30 s) | 45 ± 10                      | 46 ± 12      |
| KN-93, 0.5 μM (30 min) + carbachol (10 μM; 30 s) | 62 ± 15                      | 66 ± 10      |
| KN-93, 1 μM (30 min) + carbachol (10 μM; 30 s) | 88 ± 11                      | 108 ± 7      |
| KN-93, 2 μM (30 min) + carbachol (10 μM; 30 s) | 102 ± 11                     | 98 ± 14      |
| KN-92, 0 μM (30 min) + carbachol (10 μM; 30 s) | 36 ± 5                       | 39 ± 9       |
| KN-92, 0.1 μM (30 min) + carbachol (10 μM; 30 s) | 43 ± 10                      | 41 ± 12      |
| KN-92, 0.5 μM (30 min) + carbachol (10 μM; 30 s) | 42 ± 12                      | 41 ± 8       |
| KN-92, 1 μM (30 min) + carbachol (10 μM; 30 s) | 38 ± 7                       | 37 ± 5       |
| KN-92, 2 μM (30 min) + carbachol (10 μM; 30 s) | 40 ± 10                      | 40 ± 11      |
| KN-93, 0 μM (30 min) + UTP (10 μM; 30 s) | 48 ± 16                      |               |
| KN-93, 0.1 μM (30 min) + UTP (10 μM; 30 s) | 45 ± 11                      |               |
| KN-93, 0.5 μM (30 min) + UTP (10 μM; 30 s) | 71 ± 15                      |               |
| KN-93, 1 μM (30 min) + UTP (10 μM; 30 s) | 100 ± 9                      |               |
| KN-93, 2 μM (30 min) + UTP (10 μM; 30 s) | 99 ± 10                      |               |
| KN-62 (2 μM; 25 min) + carbachol (10 μM; 30 s) | 106 ± 12                     | 99 ± 9       |
| KN-62 (2 μM; 25 min) + carbachol (10 μM; 30 s) | 42 ± 11                      | 40 ± 11      |
| KN-62 (2 μM; 25 min) + UTP (10 μM; 30 s) | 95 ± 14                      |               |

after two washes. Astrocytes reached confluence after 7 days in culture and could be trypsinized 3 to 4 times (27). Human astrocytoma 1321N1 and rat basophilic leukemia RBL-2H3 cell lines were grown in the same complemented modified Eagle’s medium. CHO-K1 cells were grown as reported before (28). Cell culture medium, dishes, and antibiotics were from Life Technologies, Inc.

Inhibitions of Cortical Astrocytes, 1321N1 Cells, RBL-2H3 Cells, and CHO-K1 Cells—CHO-K1 cells overexpressing the human type 1 5-phosphatase were obtained as previously reported (28). When the different cell types were ∼80% confluent (7–8 × 106 cells in 8 ml of culture medium), they were washed twice with 2 ml of prewarmed KRH medium (124 mM NaCl, 5 mM KCl, 1.25 mM MgSO4, 1.45 mM CaCl2, 1.25 mM KH2PO4, 8 mM glucose, 25 mM Hepes/NaOH, pH 7.4). An aliquot of 2 ml of the same prewarmed medium containing the agent(s) was pipetted onto each culture dish. Cell incubations were terminated by aspirating the incubation medium before rapidly rinsing the cells twice with KRH medium. Cells were harvested by scraping with a rubber policeman in 200 μl of ice-cold lysis buffer (20 mM TrisHCl, pH 7.5, 150 mM KCl, 12 mM 2-mercaptoethanol, 0.5% Nonidet P-40, 100 mM NaF, 50 mM o-pheniad acid, 1 mM sodium vanadate, 0.1 mM Pefabloc, 5 μM leupeptin, and 15 μg/ml calpain inhibitors I and II). Final cell lysates were obtained by three successive cycles of freeze (in liquid nitrogen)/thaw. Ins(1,4,5)P3 and Ins(1,3,4,5)P4 5-phosphatase activities were measured at 10 and 5 μM substrate, respectively (5). Km values for Ins(1,4,5)P3 and Ins(1,3,4,5)P4 were estimated by measuring initial velocities in the presence of 0.1–50 μM substrate and by using a nonlinear least squares curve fitting of substrate-rate relationships (Marquardt-Levenberg algorithm). Sensitivity of the enzyme to magnesium ions was measured by assaying enzymic activity at 10 μM Ins(1,4,5)P3 and increasing concentrations of MgCl2 (0–50 mM). Okadaic acid, sodium orthovanadate, NaF, ATP, UTP, carbachol, histamine, tetradeacetyl phorbol acetate (TPA), Nonidet P-40, and leupeptin were from Sigma. Pefabloc was from Pentapharm. Calpain inhibitors I and II were from Roche Molecular Biochemicals. KN-93, KN-92, KN-62, calphostin C, and forskolin were from Calbiochem. KN-04 was from U.S. Biological. [3H]Ins(1,4,5)P3 and [3H]Ins(1,3,4,5)P4 were from NEN-PerkinElmer Life Sciences. SDS/PAGE, Western blotting, and immunodetection were performed as described previously (23).

Cell Labeling and Enzyme Immunoprecipitation—When cells were ∼80% confluent in 6-cm-diameter culture dishes, they were washed two
times and incubated for 2 h at 37°C in 5 ml of Dulbecco's minimum Eagle's pyrophosphate-free medium supplemented with carrier-free ortho-[32P]phosphate (1 mCi/ml; Amersham Pharmacia Biotech). The cells were subsequently washed in prewarmed KRH medium, and an aliquot of 2 ml of this medium containing the agent(s) was pipetted onto each culture dish for an incubation with agonist. Crude cell extracts were prepared as described above. Type I 5-phosphatase was immunoprecipitated using protein A-Sepharose (Amersham Pharmacia Biotech) coupled to anti-rabbit IgG (Sigma) and the rabbit polyclonal anti-human brain type I 5-phosphatase antibodies. An aliquot of 90 μl of cell extract (110 μg of protein) was immunoprecipitated in the presence of 25 μl of pretreated protein A-Sepharose and 2 μl of immune serum. Immune complexes were separated by SDS/PAGE and detected by autoradiography using a Hyperfilm-MP (Amersham Pharmacia Biotech) exposed for 24–32 h.

In Vitro Enzyme Phosphorylation by Ca2+/CaM Kinase II, PKC, and PKA—Recombinant human brain type I 5-phosphatase was affinity-purified by metal chelation as described previously (30). Phosphorylation of purified recombinant human brain type 1 5-phosphatase by Ca2+/CaM kinase II was performed at 37°C for 5 min in Hepes/NaOH, pH 7.4, 10 mM MgCl2, 1 mM ATP, 1 mM EGTA, 12 mM 2-mercaptoethanol, 0.5 μg of 5-phosphatase in the presence or absence of 25 μg of pretreated protein A-Sepharose and 2 μl of immune serum. Immune complexes were separated by SDS/PAGE and detected by autoradiography using a Hyperfilm-MP (Amersham Pharmacia Biotech) exposed for 24–32 h.

**A) Rat brain cortical astrocytes**

|          | KN-93 (μM) | KN-62 (μM) |
|----------|------------|------------|
| Cchol +  | O 1 2      | O 1 2      |
| UTP +    | O 1 2      | O 1 2      |
| KN-92 (μM) | O 1 2      | O 1 2      |
| KN-04 (μM) | O 1 2      | O 1 2      |

**B) RBL-2H3 cells**

|          | KN-93 (μM) | KN-62 (μM) |
|----------|------------|------------|
| Cchol +  | O 1 2      | O 1 2      |

FIG. 2. Okadaic acid-sensitive transient phosphorylation of type I 5-phosphatase in rat brain cortical astrocytes and RBL-2H3 cells. Cells were preincubated with ortho-[32P]P for 2 h before incubation in the presence of carbachol (Cchol) during the indicated times ("", seconds; ′, minutes) directly or after preincubation with okadaic acid (O.A.) for 30 min. Cells were lysed in the presence of protease and phosphatase inhibitors, and enzyme was immunoprecipitated using anti-type I 5-phosphatase antibodies (2 μl). The 43-kDa enzyme (indicated by an arrow) was detected by autoradiography (48-h exposure) after SDS/PAGE. Carbachol was at 10 μM, and okadaic acid was at 100 nM. The standard molecular masses (kDa) are indicated in the margin.

![Image](image_url)
itated, and washed in the presence of 75 mM phosphoric acid before counting radioactivity (31).

**Peptide Mapping and Sequence Analysis by MALDI-Reflection Time of Flight Mass Spectrometry**—The excised Coomassie Blue-stained gel band was treated as previously described (32, 33), except that the protein was denatured in 8 M urea/Tris 0.1 M, pH 8.5, and diluted to 2 M urea before tryptic digestion. Tryptic digestion was performed overnight in the presence of 500 ng of modified trypsin (Promega). Adsorption onto Poros R2 beads (PerSeptive Biosystems) as well as mass spectrometry experiments for peptide mapping and sequence analysis by PSD on a Bruker Reflex III Instrument (Bruker-Franzen Analytik GmbH) were performed as previously reported (33). Briefly, the dried peptide-bound beads were mixed with 0.7 μg of 4-hydroxycinnamic acid, 0.8 mg of 3,5-dihydroxybenzoic acid in 50% acetonitrile/water with 0.1% trifluoroacetic acid. After air-drying onto the MALDI target, MALDI mass spectra were recorded after ionization, achieved using a 337-nm nitrogen laser (attenuation between 15 and 25). In the reflectron mode, positive (monocharged) ions were accelerated to a total voltage of 26.3 kV. After selection of the interesting peptide, PSD spectra were acquired in segments, with a 15% decrease in reflectron voltage for each. PeptideSearch algorithm was used to search the non-redundant protein data base for direct peptide monoisotopic mass fingerprinting and PSD-derived sequence tag search. The Mascot algorithm (available at www.matrixscience.com) was also used for protein identification using peptide mass fingerprints and peptide fragmentation spectra.

**RESULTS**

**Identification of Type I Inositol Phosphatases by Immunodetection, Immunoprecipitation, and Mass Spectrometry**—A 43-kDa band was observed after immunodetection with anti-type I 5-phosphatase antibodies in human astrocytic 1321N1 cells, rat brain cortical astrocytes, and RBL-2H3 cells (Fig. 1A). The presence of the initial immunogenic peptide with the same immune serum during the immunodetection abolished the signal at 43 kDa (data not shown). After immunoprecipitation from a crude homogenate of 1321N1 cells and colloidal Coomassie Blue staining (Fig. 1B), the protein was excised from the gel and digested in the presence of trypsin, and the generated peptides were analyzed by mass spectrometry. Direct mass fingerprinting and sequence data obtained by PSD showed that the excised 43-kDa protein corresponded to the type I 5-phosphatase (Table I). In the supernatant obtained after immunoprecipitation from both cell types, Inositol 1,4,5-trisphosphate and Ins(1,3,4,5)P4 5-phosphatase activities were decreased by at least 90% as compared with unprecipitated enzyme (data not shown).

**Ca²⁺-raising Agents Provoked Okadaic Acid-sensitive Transient Inhibition of 43-kDa 5-Phosphatase**—Incubation of rat brain cortical astrocytes and RBL-2H3 cells with carbachol provoked a transient decrease in Ins(1,4,5)P3 and Ins(1,3,4,5)P4 5-phosphatase activities, i.e., 40–50% as compared with basal activity after 15–30 s of incubation with the agonist (Tables II and III). Maximal enzyme inactivation was achieved at 10–30 μM carbachol (Table II). The same results were obtained when stimulating the 1321N1 cells by carbachol (data...
Preincubation of rat brain cortical astrocytes and RBL-2H3 cells with okadaic acid for 30 min before receptor activation provided a maximal and more sustained inhibition of Ins(1,4,5)P3/Ins(1,3,4,5)P4 5-phosphatase, i.e. 70–85% (Tables II and III). Long time incubation with the agonist did not allow the detection of any inhibition of enzymic activity. In each cell system, inhibition of Ins(1,4,5)P3/Ins(1,3,4,5)P4 5-phosphatase was related to a decrease in \( V_{\text{max}} \) but not to a change in the apparent \( K_m \) value for Ins(1,4,5)P3 or Ins(1,3,4,5)P4 (Table IV).

Inhibition of 43-kDa 5-Phosphatase Was Prevented by Specific \( \text{Ca}^{2+}/\text{CaM} \) Kinase II Inhibitors—No modulation of Ins(1,4,5)P3 or Ins(1,3,4,5)P4 5-phosphatase activity was observed after incubation of rat cortical astrocytes, 1321N1 cells, and RBL-2H3 cells with TPA as well as after preincubation in the presence of calphostin C (a potent inhibitor of PKC) before agonist stimulation (Table IV). Forskolin did not provoke any change in 5-phosphatase activity (Table IV). However, preincubation of astrocytic cells and RBL-2H3 cells with increasing concentrations (up to 2 \( \mu \)M) of two potent \( \text{Ca}^{2+}/\text{CaM} \) kinase II inhibitors, i.e. KN-93 and KN-62, prevented agonist-mediated inhibition of Ins(1,4,5)P3 (Table V) and Ins(1,3,4,5)P4 (data not shown) 5-phosphatase activity. The two respective structural analogs KN-92 and KN-04 (inactive on \( \text{Ca}^{2+}/\text{CaM} \) kinase II) were ineffective (Table V).

Phosphorylation by \( \text{Ca}^{2+}/\text{CaM} \) Kinase II Was Necessary for Maximal Inhibition of Type I 5-Phosphatase—Rat cortical astrocytes and RBL-2H3 cells were prelabeled with ortho-\(^32\)P and incubated with carbachol to inhibit the enzymic activity. Type I 5-phosphatase was immunoprecipitated and analyzed by SDS/PAGE. Enzyme inhibition coincided with phosphate incorporation into the 43-kDa protein band (Fig. 2). Mass spectrometric analysis after excision and tryptic digestion of the radioactive 43-kDa band showed that it corresponded to type I 5-phosphatase (data not shown). \(^32\)P incorporation occurred after incubation of the two cell types with 10 \( \mu \)M carbachol for

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**FIG. 5.** *In vitro* phosphorylation of type I 5-phosphatase by \( \text{Ca}^{2+}/\text{CaM} \) kinase II. A, purified recombinant type I 5-phosphatase (0.5 \( \mu \)g) was preincubated for 5 min at 37 °C and pH 7.4 with 100 \( \mu \)M \([\gamma-\text{\(^32\)P}]\text{ATP (final activity} – 250 \mu \text{Ci/ml) in the presence of Ca}^{2+}/\text{CaM kinase II and its cofactors (lane 2, 2 \( \mu \)M CaM plus 0.5 \( \mu \)M free Ca}^{2+}; \text{lane 3, 0.5 \( \mu \)M free Ca}^{2+}, \text{PKC (and its cofactors) (lane 4), PKA (lane 5), and without any kinase (lane 6). As a control, 0.75 \( \mu \)g of purified Ins(1,4,5)P3 3-kinase A (26) was phosphorylated under the same conditions in the presence of Ca}^{2+}/\text{CaM kinase II and its cofactors (lane 1). After SDS/PAGE, the gel was stained with colloidal Coomassie Blue and then autoradiographed for 12 h. The standard molecular masses (kDa) are indicated. B, Ca}^{2+}/\text{CaM kinase II-provoked} \(^32\)P incorporation into human type I 5-phosphatase was measured by phosphorylating enzyme (5 \( \mu \)g) with Ca}^{2+}/\text{CaM kinase II (50 ng) in the presence (C) or absence (D) of 10 \( \mu \)M free Ca}^{2+}, 2 \( \mu \)M CaM, and 100 \( \mu \)M \([\gamma-\text{\(^32\)P}]\text{ATP (final activity} – 250 \mu \text{Ci/ml) for various times (0–10 min). It was also performed in the presence of PKA (E) and PKC (G) and its cofactors. After each incubation time, the enzyme was precipitated onto P81 phosphocellulose with 75 mm phosphoric acid before counting radioactivity. Results are the means of triplicates ± S.D.*
30 s. Preincubation with okadaic acid before receptor activation potentiated the phosphate incorporation into the 43-kDa enzyme in both cell systems, which was more sustained between 15 s and 1 min (Fig. 2). The same results were obtained after stimulation of human astrocytic 1321N1 cells by carbachol (data not shown). Moreover, preincubation with KN-93 or KN-62 before receptor activation prevented 32P incorporation into the enzyme in a dose-dependent manner, with a maximal effect at 2 μM. The two inactive analogs KN-92 and KN-04 did not prevent the incorporation of 32P (Fig. 3A). TPA, calphostin C, and forskolin had no effect on enzyme phosphorylation (data not shown).

In Vitro Phosphorylation and Inhibition of Recombinant 43-kDa 5-Phosphatase by Ca2+/CaM Kinase II—Since in vivo phosphorylation of type I 5-phosphatase was prevented by potent Ca2+/CaM kinase II inhibitors, we investigated in vitro phosphorylation by Ca2+/CaM kinase II of recombinant human type I 5-phosphatase produced in bacteria. Ca2+/CaM kinase II-catalyzed phosphorylation resulted in a decrease in Ins(1,4,5)P3 and Ins(1,3,4,5)P4 5-phosphatase activities in the presence of 10 μM free Ca2+ and 2 μM CaM, i.e. 50–70% as compared with basal activity measured after preincubation in the absence of Ca2+/CaM kinase II, Ca2+, and CaM. In vitro phosphorylation of type I 5-phosphatase by Ca2+/CaM kinase II did not provoke any change in sensitivity toward magnesium ions (data not shown). In agreement with previously reported data (19), PKA- and PKC-dependent phosphorylation did not provoke any modulation in Ins(1,4,5)P3 and Ins(1,3,4,5)P4 5-phosphatase activities in vitro (Fig. 4A and B). Inhibition of purified type I 5-phosphatase by Ca2+/CaM kinase II in vitro was correlated with an incorporation of 32P into the 43-kDa enzyme (Fig. 5A). This was not observed after incubation in the presence of PKA or PKC (Fig. 5A). Additionally, stoichiometric measurements indicated that the enzyme was phosphorylated by Ca2+/CaM kinase II at two major residues and reached a plateau in a time course study after 4 min at 37°C (Fig. 5B).

DISCUSSION

The apparent requirement of Ins(1,3,4,5)P4 in order for Ins(1,4,5)P3 to stimulate Ca2+ entry and mobilization has been reported in mouse lachrymal cells (34). Application of Ins(1,3,4,5)P4 in an extracellular manner in normal hippocampal slices mimicked the deterioration of ischemic neurons, so it was suggested that the formation of Ins(1,3,4,5)P4 plays a critical role in neuronal death and that Ins(1,3,4,5)P4 acts as a signal for inducing Ca2+ entry (35). In rat hippocampus, Ins(1,3,4,5)P4 enhances long term potentiation by regulating Ca2+ entry through up-regulation of voltage-gated calcium channels. So it was proposed that a possible physiological function of Ins(1,3,4,5)P4 could be to activate postsynaptic Ins(1,4,5)P3-dependent Ca2+ release and, therefore, to participate in the induction of the long term potentiation that is normally dependent on the Ca2+-induced Ca2+ release (36). In another recent study, it has been shown that the activation of the store-operated Ca2+ current Icrac in the rat mast cell line RBL-2H3 was facilitated by Ins(1,3,4,5)P3-mediated inhibition of Ins(1,4,5)P3 metabolism through type I Ins(1,4,5)P3 5-phosphatase (37). Depending on its cellular concentrations, Ins(1,3,4,5)P4 could act as a bi-modal regulator of calcium signaling, inhibiting Ins(1,4,5)P3 receptors at high concentrations and inhibiting Ins(1,4,5)P3 5-phosphatase at low concentrations by acting as a co-substrate. Such data support a second messenger function for Ins(1,3,4,5)P4 (discussed in Ref. 38).

Evidence has been provided that enzymes that phosphorylate Ins(1,4,5)P3 to Ins(1,3,4,5)P4, i.e. Ins(1,4,5)P3 3-kinase isoforms, are regulated by phosphorylation in intact cells. Ins(1,4,5)P3 3-kinase A, which is largely expressed in the dendrites and spines of hippocampal CA1 cells (39–41), is activated upon phosphorylation by Ca2+/CaM kinase II (26). Ca2+/CaM kinase II is also particularly abundant in the hippocampus, and it has been proposed that Ins(1,4,5)P3 3-kinase A may function as a co-incidence detector as it may be an effector of the “frequency reading” Ca2+/CaM kinase II (42–45). Such a regulation mechanism has also been proposed for the isoform B in primary cultures of rat cortical astrocytes and in the human 1321N1 astrocytoma cell line, where this isoenzyme could be activated through a Ca2+/CaM kinase II and protein kinase C pathway (27). The data presented here support the idea that both the Ins(1,4,5)P3 kinase and the major Ins(1,4,5)P3/Ins(1,3,4,5)P4 5-phosphatase, i.e. type I 5-phosphatase, are coordinated to generate high levels of Ins(1,3,4,5)P4. Indeed, as it has been shown for the Ins(1,4,5)P3 3-kinases A and B, we show now for the first time that type I 5-phosphatase is a target of a regulation mechanism through Ca2+/CaM kinase II in intact cells and in vitro. This was shown in rat cerebral cortex astrocytes, 1321N1 and RBL-2H3 cell lines stimulated by carbachol (as well as UTP, in the case of rat astrocytes), and in type I 5-phosphatase-transfected CHO cells stimulated by UTP. Ca2+ mobilization resulting from the increased production of Ins(1,4,5)P3 had been shown in CHO cells in response to purinoreceptor P2Y2 activation (46). Activation of P2Y2 receptor with UTP also provoked a rapid decrease in Ins(1,4,5)P3 and Ins(1,3,4,5)P4 5-phosphatase activities, i.e. 40–60% at 15–30 s with a maximal activation at 10–30 μM UTP (data not shown). The presence of type I 5-phosphatase (43-kDa protein band on SDS gel) in astrocytic cells and RBL-

| Type I Ins(1,4,5)P3 5-phosphatase | Ins(1,4,5)P3 3-kinase |
|---------------------------------|----------------------|
| Inhibition in intact cells through a Ca2+/CaM kinase II-dependent pathway in response to Ca2+-raising agonists (muscarinic, purinergic). | Activation in intact cells through a Ca2+/CaM kinase II-dependent pathway in response to Ca2+-raising agonists (muscarinic, purinergic). |
| Negative regulation observed in astrocytic and basophilic cells. | Positive regulation observed in brain cortical slices (isoform A) and astrocytic cells (isoform B). |
| Transient inhibition with a maximum at 15–30 s, protected by okadaic acid. | Transient activation with a maximum at 15–30 s, protected by okadaic acid. |
| Direct in vitro phosphorylation and inhibition by Ca2+/CaM kinase II. | Direct in vitro phosphorylation and activation by Ca2+/CaM kinase II. |
| Any effect of PKA and PKC in intact cells. | Any effect of PKA and PKC in intact cells (except for isoform B, which is activated by PKC). |
| Any effect of PKA and PKC in vitro. | Inhibition by PKC and any effect of PKA in vitro. |
2H3 cell line has been shown in this study by immunoprecipitation and mass spectrometry analysis. As for astrocytes (47), the production of inositol phosphates, especially Ins(1,3,4,5)P_4, has been established in the rat basophilic leukemia RBL-2H3 cell line (48, 49). In this cell line, the two candidate Ins(1,3,4,5)P_4 receptors GAP1/IP4BP and GAP1(m), which belong to the GAP1 family of Ras GTPase-activating proteins, have been shown to be primarily localized to the plasma membrane and to the cytoplasm, respectively (50).

When cells were preincubated with [32P]P, evidence was provided that Ca^2+/CaM kinase II inhibitors (i.e. KN-93 and KN-62) prevented phosphate incorporation into the 43-kDa protein after immunoprecipitation. Phosphorylation in both cell systems was protected in the presence of okadaic acid, suggesting that the phosphorylated enzyme could be a substrate for protein phosphatase 1 or 2A (51). Non-effective analogs of Ca^2+/CaM kinase II inhibitors, i.e. KN-92 and KN-04, did not prevent phosphorylation of the enzyme in intact cells, supporting the supposition that the effects of KN-93 and KN-62 were specific toward inhibition of Ca^2+/CaM kinase II. TPA, calphos- tin C, and forskolin did not induce any change in Ins(1,4,5)P_3 levels by decreasing type I 5-phosphatase activity nor [32P]P incorporation in any tested cell types. More directly, purified type I 5-phosphatase was phosphorylated in vitro by Ca^2+/CaM kinase II. Direct phosphorylation by Ca^2+/CaM kinase II in vitro showed that at least two residues are involved. Four consensus sites (Arg/Lys-X-X-(Ser/Thr) for protein phosphorylation by Ca^2+/CaM kinase II (52) are found in the primary structure of human type I Ins(1,4,5)P_5-phosphatase, i.e. serines 239, 310, 366, and threonine 372, and are conserved in the orthologous dog primary structure (5). These four residues are located in the catalytic domain of the enzyme, which has been so defined on the basis of the conservation of this domain sequence between the different members of the 5-phosphatase family and on the identification of critical residues involved in substrate binding and/or catalysis (50, 53–55). Phosphorylation of type I 5-phosphatase decreased enzyme activity but without affecting the sensitivity to magnesium ions (in intact cells nor in vitro).

CaM kinase II thus appears a central regulatory enzyme in phosphorylating both enzymes controlling Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 levels. It has also been reported previously that Ins(1,4,5)P_3 receptor could be a target of phosphorylation by Ca^2+/CaM kinase II, a multifunctional enzyme that converts phosphorylation of many proteins, has wide tissue distribution but is particularly abundant in brain (57). Regulation of several CaM-dependent proteins by Ca^2+/CaM kinase II has been reported. For example, phosphorylation of calcineurin decreased phosphate activity by decreasing the V_max or by increasing the K_m, depending on the substrate utilized (58). Our data suggest that in different cell types (e.g. astrocytes, Ins(1,3,4,5)P_3 levels could be controlled by Ca^2+/CaM kinase II-dependent phosphorylation of Ins(1,4,5)P_3 3-kinase and Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 5-phosphatase. Generation of oscillations in Ins(1,4,5)P_3 is thought to require Ca^2+-dependent activation of PLC (59–61). We previously reported the importance of the kinetic parameters of type I 5-phosphatase in the control of Ca^2+ oscillations in response to any extracellular signal (16, 62). Ca^2+-dependent phosphorylation of the two Ins(1,4,5)P_3- and Ins(1,3,4,5)P_4-metabolizing enzymes with opposite change in activity could provide an alternative regulatory mechanism in the generation or control of Ca^2+ oscillations (Table VI).

Recent data obtained in primary hippocampal cultures indicated that Ins(1,4,5)P_3 3-kinase A associates with F-actin and is also colocalized postsynaptically with Ca^2+/CaM kinase II (63). The data therefore suggest a direct link between the production of Ins(1,3,4,5)P_3 and calcium signaling via Ca^2+/CaM kinase II. The latter enzyme has been shown to be an integrator of pulsatile Ca^2+ signals (44). The data presented here provide an additional mechanism of control of Ins(1,3,4,5)P_3 levels by decreasing type I 5-phosphatase activity in stimulated cells. The mechanism also involves Ca^2+/CaM kinase II phosphorylation. Since type I 5-phosphatase is concentrated in neurons (6), it is tempting to speculate that both Ins(1,4,5)P_3 3-kinase and 5-phosphatase could localize in some cells and at some time. Whether type I 5-phosphatase would be associated with F-actin or be present in dendritic spines is not known. However, the enzyme can be phosphorylated (this study) and is particularly abundant in Purkinje cells, and calcium released selectively from the spine apparatus of Purkinje cells is crucial for the establishment of long term depression (64).

The coordination of the two enzymes responsible of the Ins(1,3,4,5)P_3 metabolism could be generalized to other second messengers. For example, a subset of olfactory neurons selectively express GMP-stimulated phosphodiesterase and guanylyl cyclase D and define a unique olfactory signal transduction pathway (65). Targeting of protein kinases and phosphatases to the cytoskeleton enhances the regulation of signal transduction events. Targeting of the PKA to the cytoskeleton is achieved through interaction with protein kinase A-anchoring proteins, AKAPs, which maintain multivalent signaling complexes by binding additional enzymes, including kinases and phosphatases (66). The SH2 domain-containing 5-phosphatase SHP1, when phosphorylated on tyrosine, has been shown to recruit the regulatory subunit of the phosphatidylinositol 3,4,5-trisphosphate synthetic enzyme p85 (67), arguing in favor of a coordinated action of phosphatidylinositol 3,4,5-trisphosphate synthesis and degradation.

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