Phosphorylation of GTP Cyclohydrolase I and Modulation of Its Activity in Rodent Mast Cells

GTP CYCOHYDROLASE I HYPERPHOSPHORYLATION IS COUPLED TO HIGH AFFINITY IgE RECEPTOR SIGNALING AND INVOLVES PROTEIN KINASE C*†

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GTP cyclohydrolase I controls the de novo pathway for the synthesis of tetrahydrobiopterin, which is the essential cofactor for tryptophan-5-monoxygenase and thus, for serotonin production. In mouse bone marrow-derived mast cells, the kit ligand selectively up-regulates GTP cyclohydrolase I activity (Ziegler, I., Hültner, L., Egger, D., Kempekis, B., Mailhammer, R., Gillis, S., and Rödl, W. (1993) J. Biol. Chem. 268, 12544–12551). Immunoblot analysis now confirms that this long term enhancement is caused by increased expression of the enzyme. Furthermore we show that GTP cyclohydrolase I is subject to modification at the post-translational level. In vitro labeling with 32Porthophosphate demonstrates that in primary mast cells and in transfected RBL-2H3 cells overexpressing GTP cyclohydrolase I, the enzyme exists in a phosphorylated form. Antigen binding to the high affinity receptor for IgE triggers an additional and transient phosphorylation of GTP cyclohydrolase I with a concomitant rise in its activity, and in consequence, cellular tetrahydrobiopterin levels increase. These events culminate 8 min after stimulation and can be mimicked by phorbol ester. The hyperphosphorylation is greatly reduced by the protein kinase C inhibitor Ro-31-8220. In vitro phosphorylation studies indicate that GTP cyclohydrolase I is a substrate for both casein kinase II and protein kinase C.

*(6R)-H4bipterin serves as an electron donor for the hydroxylation of aromatic amino acids and, therefore, functions as the essential cofactor for tryptophan-5-monoxygenase (EC 1.14.16.4), for phenylalanine-4-monoxygenase (EC 1.14.16.1), and for tyrosine 3-monoxygenase (EC 1.14.16.2). Consequently, these oxygenases initiate serotonin and catecholamine biosynthesis, respectively (reviewed in Ref. 1). The cofactor H4bipterin is synthesized de novo from GTP, and the first and rate-limiting step in the biosynthetic pathway is catalyzed by GTP cyclohydrolase I (EC 3.5.4.16). The subsequent activity of 6-pyruvoyl H4pterin synthase (EC 4.6.1.10) and sepiapterin reductase (EC 1.1.1.153) yields the final product, H4bipterin (reviewed in Ref. 2).

The activity of mammalian GTP cyclohydrolase I is regulated by cytokines in a cell line and tissue-specific manner. For example, in T cells (3) and macrophages (4, 5) it is induced by interferon γ (3), and in rat mesangial cells it is triggered by tumor necrosis factor α or interleukin 1β (6). Among a panel of cytokines that support the growth of murine BMMC, KL (kit ligand; or stem cell factor) selectively enhances the activity of GTP cyclohydrolase I about 6-fold (7). It was demonstrated that either interferon γ treatments of T cells and macrophages (4) or cultivation of BMMC with KL (8) increases the steady state mRNA levels of this enzyme; moreover, it was reported (6) that increased GTP cyclohydrolase I protein levels occurred in rat mesangial cells exposed to interleukin 1β.

Evidence is accumulating, however, that GTP cyclohydrolase I activity is not only subject to transcriptional or post-transcriptional regulation. For example, a conformational change may be induced by binding of a feedback regulatory protein consisting of 9.5-kDa subunits (9, 10). Moreover, the kinetics of cytokine-induced or of cell-cycle-associated GTP cyclohydrolase I activity in T cells (4) and in rat thymocytes (11), respectively, did not fully correlate with the kinetics of steady state levels of mRNA specific for the enzyme. It was suggested, therefore, that post-translational modification of GTP cyclohydrolase I may contribute to the changes in its activity. The combination of interleukin 1β with agents that elevate cellular cAMP levels caused an additive increase in mRNA for GTP cyclohydrolase I but also yielded a marked synergistic increase at the activity level in rat mesangial cells; therefore, a prominent post-translational modulation of the enzyme was postulated (6). Furthermore, the observation that phorbol ester triggers a rapid but transient accumulation of neopterin and biopterin in primed T cells and in various cell lines (12) suggested that GTP cyclohydrolase I may become phosphorylated. Finally, in PC12 cells exposed to high KCl concentrations, Imazumi et al. (13) reported that a rabphilin-3A antibody co-immunoprecipitated several phosphorylated proteins, one of which was a 30-kDa protein with a peptide map and amino acid analysis identical with GTP cyclohydrolase I. Nonetheless, a direct and unequivocal proof for a post-translational modification of this enzyme has been lacking.
Consequently, we examined phosphorylation of GTP cyclohydrolase I and its modulation of activity in response to an external stimulus in rodent mast cells. Among all primary cells of mammalian origin, the expression level of GTP cyclohydrolase I activity in KL-induced BMMC is best for a biochemical experimentation; the activity levels in these cells are 5 to 50-fold higher than in liver, adrenal, brain (14), or in primed T cells (3). On the other hand, the rat basophilic leukemia cell line RBL-2H3 has been an extensively used model for stimulus protein phosphorylation coupling in mast cells. Antigen binding to the IgE-primed cells leads to a complex sequence of events that transduce the signal. The sequence is initiated by tyrosine phosphorylation of the \( \beta \) and \( \gamma \) chains in Fc\( \epsilon \)R1 by the protein-tyrosine kinase p53/56\( \text{lyn} \) and subsequent activation of p72\( \text{syk} \) (15–17). Activation of phospholipase \( \text{C-}\gamma \) 1 by tyrosine phosphorylation possibly involves p72\( \text{syk} \) and causes phosphoinositide breakdown. This generates 1,2-\( \text{sn} \)-diacylglycerol as a potent activator of all PKC isotypes (except PKC-\( \zeta \)) that are reported to occur in rodent mast cells (reviewed in Refs. 16 and 17). The \( \delta \) isoform of PKC partly associates with and phosphorylates the receptor upon engagement of Fc\( \epsilon \)R1 by antigen. Besides, PKC-\( \delta \) becomes phosphorylated on tyrosine residues associated with the membrane and primed to phosphorylate various substrates (Refs. 18 and 19; reviewed in Ref. 16).

Here we show that the activity of GTP cyclohydrolase I is rapidly and transiently enhanced in response to antigen binding to Fc\( \epsilon \)R1 of KL-induced BMMC and of RBL-2H3. Furthermore, we demonstrate that the enzyme exists in the cell in a phosphorylated form and that it becomes hyperphosphorylated upon antigen triggering. Evidence is provided that PKC is linked to the modulation of enzyme phosphorylation and activity and thus, to a rapid regulation of \( \text{H}_4 \)-bioppterin production of the mast cell.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—The sources of all reagents that were used for cell cultivation (7, 20) as well as for determination of enzymatic activities and for protein and biopterin determination (7, 21–24) have been described previously. The sources of the following reagents appear in parentheses; phorbol ester (PMA) (Sigma); Ro-31-8220 and hemocyanin-DNP conjugate (Calbiochem); 5,6-dichloro-1-\( \beta \)-D-ribofuranosyl-

**FIG. 1. Effect of interleukin 3 and of KL on GTP cyclohydrolase I protein expression in BMMC.** Cells were cultured with interleukin 3 or additionally exposed to KL for 40 h. After lysis, cell extracts were separated by SDS-PAGE and transferred to nitrocellulose membrane. GTP cyclohydrolase I was detected on the blot by probing with the mAb MGTP-6H11 as described under "Experimental Procedures." A, recombinant rat GTP cyclohydrolase I overexpressed in E. coli. B, BMMC cultured with KL. C, BMMC cultured without KL. One of three representative independent experiments is shown. GCH I, GTP cyclohydrolase I.

**FIG. 2. Modulation of GTP cyclohydrolase I activity and cellular biopterin levels in BMMC and in RBL-2H3 in response to Fc\( \epsilon \)R1 activation.** Comparison with the mastocytoma line P815 lacking the \( \alpha \) and \( \beta \) chains of Fc\( \epsilon \)R1 is shown. BMMC were induced for optimum GTP cyclohydrolase I expression by culturing for 40 h with KL. They were primed by incubation with monoclonal anti-DNP IgE and then stimulated with DNP conjugated with hemocyanin for varying times. The specific activities of GTP cyclohydrolase I were measured at t\( _0 \) and normalized; the percentage values are the mean \( \pm \) S.E. The cellular biopterin levels were 276.7 \( \pm \) 138.5 pmol mg\( ^{-1} \) (BMMC) and 51.3 \( \pm \) 18.6 pmol mg\( ^{-1} \) (RBL-2H3 cells). For calculation of the kinetics, the actual specific activities were normalized; the percentage values are the mean \( \pm \) S.E. \( \text{H}_4 \)-bioppterin levels in P815 cells were close to detection limits; they are therefore inappropriate to determine kinetics. A, BMMC. B, RBL-2H3. C, P815. ○, with antigen trigger; ●, controls (priming with IgE was omitted). Upper panel: GTP cyclohydrolase I activity; lower panel: cellular biopterin.
benzimidazole (Biomol); protein G-Sepharose Fast Flow (Amersham Pharmacia Biotech); ECL detection system, ECL biotinylation system, Hybond nitrocellulose membrane (Amersham); CNBr-activated Sepharose (Amersham); alkaline phosphatase (Boehringer Mannheim). Antibodies were obtained from the following sources: anti-rat IgG antibodies for immunoblotting (Zymed Laboratories Inc. and Bio-Rad, Richmond, CA); Type Culture Collection); peroxidase-conjugated anti-rat IgG and second antibodies (Dianova); monoclonal IgG antibody to DNP (Sigma); Dulbecco’s modified Eagle’s medium and supplements (Life Technologies); myelin basic protein (Life Technologies, Inc.); green fluorescent protein vector (CLONTECH).

Generation of Monoclonal Antibodies to Murine GTP Cyclohydrolase I—Monoclonal antibodies were generated by immunizing Lou/C rats with a bacterially expressed murine GTP cyclohydrolase I fused in frame with Escherichia coli maltose binding protein (MGTP-maltose binding protein) (25). The procedure was essentially as described in Kremmer et al. (26). Screening of hybridoma supernatants was performed in a solid-phase immunoassay using bacterial extracts from E. coli expressing either the MGTP-maltose binding protein or a maltose binding protein control. The immunoglobulin type was determined with rat Ig class (anti-IgM) and IgG subclass-specific mouse mAbs. Antibody specificity of MGTP-6B6 (rat IgG2b) and MGTP-6H11 (rat IgG2a) was tested by immunoblotting against murine GTP cyclohydrolase I expressed in E. coli (see Fig. 1). The monoclonal antibodies were purified using Protein G-Sepharose columns. In immunoprecipitates obtained from extracts of KL-induced BMMC or from E. coli expressing recombinant GTP cyclohydrolase I, 90% of total GTP cyclohydrolase I activity was recovered. Moreover, pre-saturation of the mAb MGTP-6B6 with recombinant rat GTP cyclohydrolase I inhibited the subsequent immunoprecipitation as examined by activity determination in immunoprecipitates of KL-induced BMMC.

Cell Culture and Cell Stimulation—Primary mouse BMMC were obtained from femoral bone marrow and were kept in interleukin 3-dependent growth. Their maturation was induced by KL for 40 h. Details of the culture conditions have been described previously (7, 20). P815 cells were obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig. The 2H3 subline of RBL cells was a gift of D. Arndt-Jovin, Goettingen, Germany. Cells were maintained in flasks with RPMI 1640 medium supplemented with 10% fetal bovine serum in a 5% CO2 atmosphere. For experiments, they were seeded in 60-mm Petri dishes and grown to a density of 1–5 × 10⁶ cells/dish. The cells were loaded with monoclonal anti-DNP IgE (0.5–1 µg/ml; 2–4 h). After washing in Hank’s solution, activation with DNP conjugated to hemocyanin (1–3 µg/ml) or with 60 nM PMA was performed for 37 °C at the indicated time according to standard procedure (27, 28). The reaction was terminated by freezing of the cell pellet (BMMC and P815) or the Petri dishes (RBL-2H3) in liquid nitrogen.

Overexpression of GTP Cyclohydrolase I in RBL-2H3 Cells—An EcoRI/HindIII fragment of pNCO-GTP (25, 29), which comprises the entire rat GTP cyclohydrolase cDNA sequence, had been cloned into the pBS1 vector (30), yielding pSBC1. The construct was cotransfected into RBL-2H3 cells. RBL-2H3 cells were adjusted to 10⁸ cells/ml in X-tot medium, and 10⁷ cells were plated in 10-cm-diameter plates of 100-mm diameter. Primary mouse BMMC were induced for optimum GTP cyclohydrolase I expression by culturing with KL for 40 h. IgE-sensitized cells were preloaded with [³²P]orthophosphate and then activated for varying times with antigen. GTP cyclohydrolase I was isolated from the lysates by immunoprecipitation using the mAb MGTP-6B6 coupled with CNBr-activated Sepharose. The immunoprecipitates were extensively washed with lysis buffer and resolved by SDS-PAGE. A, immunoblot probed with the mAb MGTP-6H11 (see Fig. 1); i.c., isotype control; rec. GCH I, recombinant GTP cyclohydrolase I expressed in E. coli. B, autoradiography. C, quantification of GTP cyclohydrolase I phosphorylation (relative units) using phosphoimaging. One of three representative independent experiments is shown.

Cell Solubilization, Immunoprecipitation, and Immunoblotting—Cells (0.8–1 × 10⁶) were lysed, and GTP cyclohydrolase I was immunoprecipitated by MGTP-6B6 mAb and coupled with CNBr-activated Sepharose according to the manufacturer’s instructions. The procedures of lysis and precipitation were essentially the same as described previously (20). Depending on the experiment, precisions and precipitations with control antibodies were performed and are indicated in the legend. After solubilization of the immunoprecipitates, equal amounts of protein were resolved by 10% SDS-PAGE under reducing conditions and immediately transferred to nitrocellulose membranes. Membranes were blocked, probed with the biotinylated MGTP-6H11 mAb, and visualized using peroxidase-conjugated streptavidin and enhanced chemiluminescence. [³²P]labeling of GTP cyclohydrolase I was documented by autoradiography of the blotting membrane to a Kodak X-Omat AR film. Relative quantitative estimations of radioactivity were performed with Fuji BAS1000 phosphorimaging. Nolabeled extracts were blotted and then probed with purified MGTP-6H11 mAb, followed by second stage peroxidase-conjugated anti-rat IgG and ECL detection.

In Vitro Phosphorylation of GTP Cyclohydrolase I in Immune Complex Kinase Assays—Recombinant rat GTP cyclohydrolase I from overexpression E. coli and RBL-2H3 cells was immunoprecipitated using Sepharose-coupled MGTP-6B6 mAb. The bead-bound GTP cyclohydrolase I immune complexes were washed 2 times with lysis buffer, resuspended in kinase buffer (33), and washed 3 times with 500 μl of the same buffer. The kinase reaction was performed for 30 min at 30 °C essentially as described (33). Recombinant PKC isoforms α, β, and δ purified from baculo virus-infected insect cells (34) were adjusted to equal activity using myelin basic protein as a substrate. The reactions were stopped by the addition of SDS sample buffer, and the proteins were resolved on a 10% SDS-PAGE and blotted on nitrocellulose membranes. The labeled substrates were visualized by autoradiography after probing with MGTP-6H11 antibody as described above.

Extraction of the Cells and HPLC Determination of Neopterin and Biopterin—The cells were homogenized in Tris buffer (50 mM, pH 8.0) containing 2.5 mM EDTA. Aliquots of the centrifuged extracts (15 min at 14 000 × g) were used for determination of pterins and enzyme assays. H₂-neopterin and H₂-biopterin were determined after acidic ionone oxidation of the reduced forms. Deproteinization by trichloroacetic acid, prepurification by cation exchange chromatography, separation by reverse-phase HPLC, and fluorometric detection have been described previously (21). The modifications of the method were the same as detailed in (3, 21–24).
independent experiments is shown.

cyclohydrolase I.

Cells were transfected with rat GTP cyclohydrolase I as described under “Experimental Procedures.” Priming of the transfected RBL-2H3 cells.

For determination of 6-pyruvoyl-H4pterin synthase, dihydroneopterin terin was then determined by reverse-phase HPLC as described above.

The specific activities of GTP cyclohydrolase I in the transfectants at $t$ of percentage values see the Fig. 2 legend; *, $p < 0.05$.

This confirms that GTP cyclohydrolase I represents a key enzyme in the rate-limiting step in both BMMC and the rat mast cell line RBL-2H3. With BMMC, the activities of 6-pyruvoyl-H$_4$pterin synthase and sepiapterin reductase had earlier been found to largely exceed the activity of GTP cyclohydrolase I (7). In RBL-2H3, the activity of GTP cyclohydrolase I was much lower than in BMMC (for activity levels, see the Fig. 2 legend). Similarly, in RBL-2H3 cells, the activities of the two subsequent enzymes in the de novo pathway of H$_4$biopterin synthesis were 16- to 20-fold higher than GTP cyclohydrolase I activity (data not shown). This confirms that GTP cyclohydrolase I represents the rate-limiting step in both BMMC and the rat mast cell line.

The BMMC and RBL-2H3 cells were sensitized with an anti-DNP monoclonal IgE and stimulated with antigen. The activity of GTP cyclohydrolase I in the presence of KL increases the activity of GTP cyclohydrolase I more than 6-fold. Enzyme activities and H$_4$biopterin production culminate after 40 h as described earlier (7).

Expression of GTP Cyclohydrolase I—Cultivation of BMMC in the presence of KL increases the activity of GTP cyclohydrolase I more than 6-fold. Enzyme activities and H$_4$biopterin production culminate after 40 h as described earlier (7). The cellular expression levels of the enzyme in extracts of interleukin 3-grown cells and of cells exposed to KL for 40 h were compared by immunoblotting. The blots were probed with either mAb MGTP-6H11 (Fig. 1) or mAb MGTP-6B6 (data not shown). The comparison with recombinant murine GTP cyclohydrolase I confirmed the specificity of both mAbs; the blots showed that cultivation with KL markedly enhances the expression of the GTP cyclohydrolase I (Fig. 1). The experiments, moreover, clearly demonstrate that such high expression levels of GTP cyclohydrolase I make KL-induced BMMC an appropriate tool to investigate regulation at the protein level.

Fig. 4. Antigen-induced modulation of GTP cyclohydrolase I activity and hyperphosphorylation of the enzyme in transiently transfected RBL-2H3 cells. Cells were transfected with rat GTP cyclohydrolase I as described under “Experimental Procedures.” Priming of the cells with IgE, antigen stimulation, determination of enzyme activity, [32P]orthophosphate incorporation, immunoprecipitation, and immunoblott ing were the same as described for BMMC (Fig. 3). A, GTP cyclohydrolase I activity in the transfectants in response to antigen binding to FceRI. The comparison with recombinant GTP cyclohydrolase I, C, autoradiography. D, quantification of GTP cyclohydrolase I phosphorylation (relative units). One of three representative independent experiments is shown.
In KL-induced BMMC, the cofactor concentration is calculated to be about 30 μM (7). With both the nonneuronal and neuronal enzyme, the in vitro $K_m$ value for H$_4$bipterin is 22–28 μM (37); the increases in cellular H$_4$bipterin levels to about 50 μM, induced by FcεRI triggering, occur close to the $K_m$ values and thus will result in significant changes of tryptophan 5-mono-oxygenase activity.

Hyperphosphorylation of GTP Cyclohydrolase I Mediated by FcεRI Signaling—The expression of GTP cyclohydrolase I was induced in BMMC by KL (see Fig. 1), and the cells were metabolically labeled with $^{32}$P. These cells were IgE-primed and stimulated with antigen. GTP cyclohydrolase I was immunoprecipitated from the lysate with the mAb MGTP-6B6. After separation by SDS-PAGE, probing of the blots with the mAb MGTP-6H11 clearly identified the major phosphorylated protein as GTP cyclohydrolase I (Fig. 3, A and B). It initially exists in IgE primed and unprimed cells in a phosphorylated form. As expected, no changes in GTP cyclohydrolase I protein levels occurred, whereas the phosphorylation transiently increased (Fig. 3, A and C). This hyperphosphorylation culminated 8–12 min after antigen stimulation. In addition to GTP cyclohydrolase I, the mAb MGTP-6B6 co-precipitated phosphorylated proteins that were not further characterized in this study.

Modulation of GTP Cyclohydrolase I Activity and Phosphorylation in Transiently Transfected RBL-2H3 Cells Overexpressing the Enzyme—The results of co-transfecting pSB1-GTP coding for GTP cyclohydrolase I and pUH1D15–1 coding for rtTA transactivator protein was examined by immunoblotting, by determination of GTP cyclohydrolase I activity, and by determination of H$_4$bipterin production by the cells; they demonstrated a 10- to 50-fold increase in all these parameter levels as compared with RBL-2H3.

After priming with IgE and stimulation with antigen, the transiently transfected cells showed a marked increase in GTP cyclohydrolase I activity (Fig. 4A). Likewise to BMMC, the enzyme, immunoprecipitated from cells that had been labeled in vivo with $[^{32}P]$orthophosphate, was found to be present in the cells as a $[^{32}P]$-labeled phosphorylated protein (Fig. 4, B and C). This confirms the results showing that GTP cyclohydrolase I initially exists as a phosphorylated enzyme in BMMC. Also, as in BMMC, antigen binding to FcεRI leads to a transient increase in GTP cyclohydrolase I phosphorylation without a change in enzyme protein levels (Fig. 4, B and D). In the transfectants, antigen stimulation caused a higher enhancement of GTP cyclohydrolase I activity and of its phosphorylation status than in BMMC.

In Vitro Phosphorylation of GTP Cyclohydrolase I by PKC-δ and Casein Kinase II—A Protease data base search revealed putative phosphorylation sites for PKC and casein kinase II that are highly conserved throughout eucaryotic GTP cyclohydrolases I including human, rat, mouse, chicken, and fish (38 see also Discussion)). To test the ability of PKC isozymes and of casein kinase II to phosphorylate GTP cyclohydrolase I, we carried out in vitro phosphorylation experiments using GTP cyclohydrolase I immunocomplexes as a substrate and the purified PKC isoforms α, β1, and δ and casein kinase II. The PKC isoforms used in these experiments were adjusted to equal activity levels using myelin basic protein as a substrate. The substrate, GTP cyclohydrolase I, was overexpressed and immunoprecipitated either from E. coli or from RBL-2H3 cells. The PKC isoforms α and β1 showed only a minor phosphorylation capacity, but in contrast, PKC-δ, under our experimental conditions, catalyzed the phosphorylation of GTP cyclohydrolase I. The potent PKC inhibitor Ro-31-8220 (IC$_{50}$ = 10 nM (39)) also markedly reduced this in vitro phosphorylation of the enzyme at concentrations of 10 nM (Fig. 5). Casein kinase II also phosphorylated GTP cyclohydrolase I with an efficiency comparable with the autophosphorylation of its own smaller subunit. The casein kinase II inhibitor 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole, consistent with its low inhibitory capacity (IC$_{50}$ = 6 μM (40, 41)), reduced the phosphorylation of GTP cyclohydrolase I by approximately 50% at concentrations of 10 μM (Fig. 5). The data from these in vitro phosphorylation experiments indicate that GTP cyclohydrolase I, expressed by both E. coli and RBL-2H3 cells, is likely to be phosphorylated by both kinases.

Enhancement of GTP Cyclohydrolase I Activity and Phosphorylation by PMA and Inhibition by Ro-31-8220—The putative involvement of PKC in the activation of GTP cyclohydrolase I and in its hyperphosphorylation was further examined by the use of PKC activators and inhibitors. First, PMA, which mimics the generation of diacylglycerol (42), effectively modulated GTP cyclohydrolase I activity in RBL-2H3 cells (Fig. 6A). The putative phosphorylation sites for PKC and casein kinase II (GCH I) by PKC isoforms and casein kinase II (CK-II). GTP cyclohydrolase I from overexpressing RBL-2H3 cells was immunoprecipitated with Sepharose-coupled MGTP-6B6 mAb, and the immunocomplexes were extensively washed, resuspended in kinase buffer, and incubated with the kinase and the inhibitors (Ro-31–8220, 10 nM; 5,6-dichloro-1-β-d-ribofuranosylbenzimidazole, 10 μM, respectively) as described under “Experimental Procedures.” Activities of the PKC isozymes were previously determined with myelin basic protein as substrate and adjusted to equal activities. The phosphorylated polypeptides of M, 26 and 44 kDa correspond to the autophosphorylated subunits of casein kinase II. A, immunoblot. B, autoradiography. One of four representative independent experiments is shown. rel. Units, relative units.
DISCUSSION

Previous studies showed that KL regulates H₄biopterin synthesis through modulation of GTP cyclohydrolase I activity in BMMC (7). We now confirm that this cytokine-induced increase in activity, culminating after 40 h, is due to increased levels of GTP cyclohydrolase I. To the best of our knowledge, we also present for the first time evidence that GTP cyclohydrolase I is additionally subject to short term modifications at the post-translational level. By development of monoclonal antibodies suitable for either immunoblotting or for immunoprecipitation, respectively, we demonstrate that the enzyme is present as a phosphorylated protein in the mammalian cell. In both KL-induced BMMC and in RBL-2H3 cells overexpressing GTP cyclohydrolase I, the enzyme undergoes additional phosphorylation when FcεRI aggregation has initiated a signal cascade resulting in a stimulation of serine, threonine, and tyrosine phosphorylation of cellular proteins. This hyperphosphorylation is transient and culminates after 8 min. Concomitantly, the activity of the enzyme is modulated in response to FcεRI aggregation and results in transiently increased cellular H₄biopterin levels.

The amino acid sequence of GTP cyclohydrolase I is highly conserved; the amino acid sequences that are essential for catalysis of the *E. coli* enzyme (43) are identical in all of the 15 unrelated species compared (38). Furthermore, the mammalian enzyme possesses identical sequences at the proposed sites for phosphorylation by casein kinase II ((S/T)XX(D/E) (44)) and by PKC ((S/T)X(R/K) (45). Prosite data base searches have revealed the conserved casein kinase II sites at positions 14, 51, 82, 103, 131, and 231 and a PKC site at position 167 (numbering is according to mouse GTP cyclohydrolase I sequence (38)). Under our experimental conditions, GTP cyclohydrolase I appears to be a substrate for both kinases, and among the PKC isoforms tested, PKC-δ is the most effective one. Quantitative data will have to compare this substrate with classical substrates for PKC-δ and thus unequivocally prove its specificity. *In vitro* studies, moreover, do not necessarily identify the kinase being involved in the basal phosphorylation or the hyperphosphorylation of GTP cyclohydrolase I under *in vivo* conditions.

To examine these reactions *in vivo*, our experiments did not further investigate the basal phosphorylation but concentrated on the hyperphosphorylation of GTP cyclohydrolase I, which is clearly part of the signal cascade initiated by FcεRI stimulation. PMA, which activates PKC in a similar manner to diacylglycerol (42), can mimic the antigen-triggered phosphorylation of the enzyme. This additional (hyper)phosphorylation is almost completely abrogated by Ro-31-8220, a selective PKC inhibitor (39). These data collectively indicate that PKC is essentially involved in the *in vivo* process of antigen-induced phosphorylation of GTP cyclohydrolase I and that the δ isoform is involved in vivo. It is well established that PKC-δ is critical to the effector function of the mast cell and that it associates with the FcεRI β-chain and phosphorylates FcεRI γ-chain in response to antigen binding (28). In this way, PKC-δ may interact with p53/p56lyn (19) and become tyrosine-phosphory-
lated (18). The phosphorylation of PKC-δ decreases the activity toward the receptor γ chain and modifies its specificity to include new substrates (18). Our results are consistent with the view that GTP cyclohydrolase I may represent one of these new substrates.

Further studies are needed to verify the sites and pathways for the antigen-induced hyperphosphorylation of GTP cyclohydrolase I and to consider possible associations with other phosphorylated proteins that coprecipitated with GTP cyclohydrolase I in primary BMMC under our experimental conditions. The rapid reversal of hyperphosphorylation indicates that protein phosphatases are also involved that need to be identified as further important components in the process of GTP cyclohydrolase I modification.

The time course of GTP cyclohydrolase I hyperphosphorylation correlates closely with modulation of its activity. Similar to other cellular systems (3), this first enzyme specific to H4biopterin synthesis proved to be the rate-limiting step in mast cells, and consequently, cellular H4biopterin levels increased upon their antigen stimulation. Since these fluctuations in cellular H4biopterin levels take place around the K_m value of tryptophan 5-monooxygenase for its cofactor, they occur in the most effective range. The rapid decline of the pterin levels following 8 min of stimulation (Fig. 2) appears to be caused by enzyme-catalyzed degradation rather than by secretion and/or auto-oxidation. Neither in the cells nor in the culture medium could the typical products of H4biopterin auto-oxidation such as 6-carboxypterin (46) or 6-hydroxypterin, be detected (data not shown). Pterin deaminase (EC 3.5.4.11), converting pterins to the corresponding lumazines, has been partially purified from both bacterial (47) and mammalian (48) sources. The activity of this enzyme is controlled by cAMP in Dictyostelium (49). However, this candidate enzyme for the additional control of H4biopterin levels during allergic mast cell response is currently only poorly characterized. In conclusion, these observations on post-translational modification of GTP cyclohydrolase I advance our knowledge of the short term regulation of H4biopterin production, but there is still much to be done to elucidate the inter-relations existing between all the components of the system and their functional role and importance for the coupling of the biosynthesis, degradation, and release of serotonin or catecholamines in living cells.

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