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Corticotropin-releasing factor induces phosphorylation of phospholipase C-γ at tyrosine residues via its receptor 2β in human epidermoid A-431 cells

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Abstract

This laboratory previously reported that corticotropin-releasing factor (CRF) increased intracellular free calcium concentrations, cellular cAMP, inositol 1,4,5-trisphosphate, protein kinase C activity, and protein phosphorylation in human A-431 cells. The increase was blocked by CRF receptor antagonist. In this study, we identified the type of CRF receptors present and investigated whether CRF induced tyrosine phosphorylation of phospholipase C-γ via CRF receptors. Using novel primers in reverse transcriptase-polymerase chain reaction, we determined the CRF receptor type to be that of 2β. The levels of the CRF receptor type 2β were not altered in cells treated with activators of protein kinase C, Ca^{2+} ionophore, or cells overexpressing heat shock protein 70 kDa. Cells treated with CRF displayed increases in protein tyrosine phosphorylation approximately at 150 kDa as detected by immunoblotting using an antibody against phosphotyrosine. Immunoprecipitation with antibodies directed against phospholipase C-β3, -γ1, or -γ2 isoforms (which have molecular weights around 150 kDa) followed by Western blotting using an anti-phosphotyrosine antibody showed that only phospholipase C-γ1 and -γ2 were phosphorylated. The increase in phospholipase C-γ phosphorylation was concentration-dependent with an EC_{50} of 4.2 ± 0.1 pM. The maximal phosphorylation by CRF at 1 nM occurred by 5 min. The CRF-induced phosphorylation was inhibited by the protein tyrosine kinase inhibitors genistein and herbimycin A, suggesting that CRF activates protein tyrosine kinases. Treatment of cells with CRF receptor antagonist, but not pertussis toxin, prior to treatment with CRF inhibited the CRF-induced phosphorylation, suggesting it is mediated by the CRF receptor type 2β that is not coupled to pertussis toxin-sensitive G-proteins. Treatment with 1,2-bis(2-iminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid attenuated the phospholipase C-γ phosphorylation. In summary, CRF induces phospholipase C-γ phosphorylation at tyrosine residues, which depends on Ca^{2+} and is mediated by activation of protein tyrosine kinases via the CRF receptor type 2β.

Keywords: CRF (corticotropin-releasing factor); CRF receptor; Ca^{2+}; Phosphorylation; Protein tyrosine kinase; G-protein; Pertussis toxin; (Human); Epithelium; PCR (polymerase chain reaction); Protein kinase C; Heat shock protein 70 kDa

1. Introduction

Corticotropin-releasing factor (CRF) orchestrates endocrine, behavioral, and autonomic responses to stress in addition to regulating release of adrenocorticotropin and proopiomelanocortin. This laboratory has previously reported that CRF inhibits protein extravasation in rats after antidromic neurogenic stimulation (Kiang and Wei, 1985), thermal injury (Kiang and Wei, 1987), and other assaults (Wei and Kiang, 1987). Culture of human epidermoid A-431 cells in the presence of CRF results in increased cellular cAMP levels, intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), inositol 1,4,5-trisphosphate (Kiang, 1994). In addition, CRF increases protein kinase C activity and its translocation from cytosol to plasma membrane (Kiang et al., 1994), and phosphorylation of cytosolic proteins at tyrosine residues (Kiang, 1997). The CRF-induced increase in $[Ca^{2+}]_i$ is the result of activation of receptor-operated Ca^{2+} channels coupled to pertussis toxin-sensitive G proteins (Kiang, 1994) and of Ca^{2+} mobilization from inositol 1,4,5-trisphosphate-sensitive pools via activation of protein tyrosine kinases (Kiang, 1997). Increases in $[Ca^{2+}]_i$ induced by CRF correlate with the ability of CRF to inhibit edema and protein extravasation caused by thermal injury in rats (Kiang, 1997).

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Three types of CRF receptors (1, 2α, and 2β) have been shown to be present in cells (Chen et al., 1993; Perrin et al., 1993; Lovenberg et al., 1995a,b), and their amino-acid sequences have been determined (Perrin et al., 1993, 1995; Lovenberg et al., 1995a). In this study, we used reverse transcriptase-polymerase chain reaction (RT-PCR) and determined that the CRF receptors present in A-431 cells were 2β and not altered by the protein kinase C activator phosbol 12-myristate 13-acetate (PMA), the Ca^{2+} ionophore ionomycin, or the overexpression of heat shock protein 70 kDa (HSP-70).

Since it is known that CRF increases protein phosphorylation at tyrosine residues (Kiang, 1997) and γ isoforms of phospholipase C (PLC) are not coupled to G proteins and are activated by phosphorylation, we investigated whether CRF induced tyrosine phosphorylation of PLC-γ isoform. In this paper, we report that CRF phosphorylated PLC-γ isoform through the CRF receptor type 2β by activating protein tyrosine kinases, which was dependent on the level of $[Ca^{2+}]_i$.

2. Materials and methods

2.1. Cell culture

A-431 cells (American Type Culture Collection, Rockville, MD) were grown in 150 cm^2 flasks in an incubator at 37°C (with 5% CO_2 in air). Cells were maintained in Dulbecco's modified Eagle culture medium containing 0.03% glutamine, 4.5 g l^{-1} glucose, 25 mM HEPES, 10% fetal bovine serum, 50 μg ml^{-1} penicillin, and 50 U ml^{-1} streptomycin (Gibco BRL, Gaithersburg, MD).

2.2. Gene transfection

The HSP-70 cDNA used in this study was provided by Dr. Richard I. Morimoto (Hunt and Morimoto, 1985). The full-length HSP-70 cDNA (2400 bp) was inserted into plasmid pH 2.3 using *Bam*HI and *Eco*RI restriction endonucleases. The transfection vector was pcDNA3 (5.4 kilobases; InVitrogen, San Diego, CA), which contains enhancer-promoter sequences from human cytomegalovirus. T4 DNA ligase was used to ligate the gene within the pcDNA3 vector, and plasmids were transformed into competent *Escherichia coli* DH5a cells (Gibco BRL) for amplification. The presence of the HSP-70 cDNA in *E. coli* was verified by PCR.

Transfection of the HSP-70 cDNA into cells was conducted in 60-mm petri dishes (50%–60% confluence; Calcium Phosphate Transfection System, Gibco BRL). Cells were incubated with plasmid DNA (15 μg; final concentration: 3 μg ml^{-1} [10^6 cells]$^{-1}$) or vector alone in HEPES-buffered saline solution containing 0.75 mM $NaHPO_4 \cdot 2H_2O$ and 12 mM $CaCl_2$ at 37°C (16 h), then washed twice before incubation in normal media (48 h). Transfected cells were selected by subsequent incubation with 200 μg ml^{-1} geneticin (G418). RT-PCR and Western-blot analysis verified successful transfection (Kiang et al., 1998).

2.3. RT-PCR

Cells (5×10^6) were trypsinized, and RNA was isolated using a commercial kit (RNA SATA-60™, TEL-TEST 'B', Friendswood, TX). RNA was quantified spectrophotometrically. The method to perform RT-PCR reaction has been published elsewhere (Kiang et al., 1996b).

The CRF genes used were CRF receptor type 1 (CRF-R1), cloned by Chen et al. (1993) and Perrin et al. (1993) and CRF receptor type 2 (CRF-R2), cloned by Lovenberg et al. (1995a). Primers used were:

CRF-R1	5′-TGT CCA CCT CCC TTC AGG AT-3′
	5′-TGA CTG CAA CAT GGT AGT GT-3′
CRF-R2	5′-CCG AAT ACT TCA ACG GCA TC-3′
	5′-AAG CAG GAA AGC AGC CAC CA-3′
CRF-R2α	5′-ATG GAC GCG GCA CTG CTC CA-3′
	5′-GGG CTC ACA CTG TGA GTA GT-3′
CRF-R2β	5′-ATG AGG GGT CCC TCA GGG CC-3′
	5′-CTG AGA GGT TGG TGA GGG TC-3′
β-actin	5′-CAT GGG TCA GAA GGA TTC CT-3′
	5′-AGC TCG TAG CTC TTC TCC AG-3′

The PCR products encoding CRF-R1, -R2, -R2α, -R2β, and β-actin mRNA were 300, 218, 300, 185, and 584 bp, respectively.

2.4. Measurements of $[Ca^{2+}]_i$

Confluent monolayers of cells were loaded with 5 μM fura-2 acetomethyl ester (fura-2AM) (Molecular Probes, Eugene, OR) plus 0.2% pluronic F-127 at 37°C (60 min), then washed with Na^+ Hanks' buffer before fluorescence measurements, as described previously (Kiang, 1994).

2.5. Immunoprecipitation and Western blots

Monolayers of cells (5×10^6) were removed from petri dishes by trypsinization and resuspended in lysis buffer containing 10 mM Tris, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 30 mM Na pyrophosphate, 0.1 mM Na orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreotol, 10 μg ml^{-1} leupeptin, 5 μg ml^{-1} aprotinin, and 1% Triton, pH 7.4. The suspension was sonicated, centrifuged at $12\,466 \times g$ for 10 min at 4°C, and then the supernatant was collected. The protein content was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, South Richmond, CA). Each sample containing 300 μg protein was used to incubate with the specified antibody (5 μl), chilled on ice for 1 h, then added 50 μl protein A/G agarose, and incubated overnight on a nutator at 4°C. The immunoprecipitate was collected with centrifu-

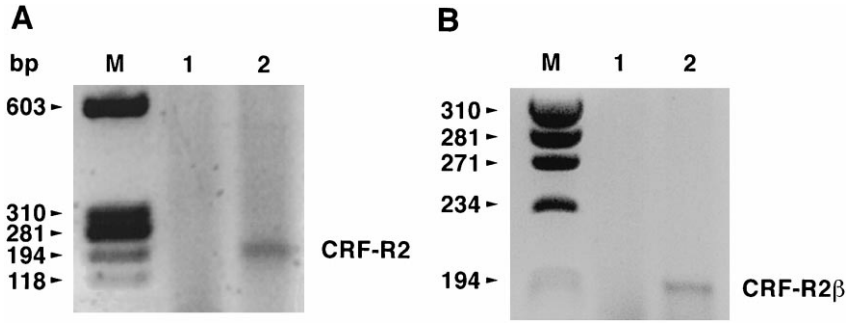

Fig. 1. Expression of CRF-R2β mRNA in A-431 cells. RT-PCR was performed as described in Section 2. 'M' represents ϕX174/*Hae*III markers. Three independent experiments were performed. (A) Representative gel of CRF-R2 mRNA expression in RNA extracted from untreated A-431 cells. (B) Representative gel of CRF-R2β mRNA expression in RNA extracted from untreated A-431 cells.

gation at $12\,466 \times g$ for 10 min, washed with 500 μl stop buffer (same as lysis buffer without 1% Triton) twice and 500 μl Tris wash buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, pH 7.4) once. The pellet was resuspended in the 50 μl sample buffer (10% sodium dodecyl sulfate) without 2-mercaptoethanol, boiled for 5 min, spun for 30 s to remove agarose. The supernatant was incubated with 5% 2-mercaptoethanol at 37°C for 1 h. Twenty-five microliters of the sample was loaded onto precasted 10% tris-glycine polyacrylamide gels (Novex, San Diego, CA). After electrophoresis, the separate proteins were transferred to a nitrocellulose membrane (MSI Micron Separations, West Borough, MA) using a Novex blotting module. Phosphotyrosine was detected using an anti-phosphotyrosine antibody and the manufacturer's protocol with enhanced chemiluminescence kit. The amount of phosphotyrosine was quantitated after background correction by densitometry.

2.6. Statistical analysis

All data are expressed as mean ± S.E.M. Analysis of variance, Studentized range test, and Student's *t*-test were used for comparison of groups with $P < 0.05$ as the significant level. Curve fitting was performed and median effective concentrations (EC_{50}) were calculated using the Inplot program (GraphPad, San Diego, CA).

2.7. Chemicals

CRF and α-helical CRF-(9–41) were purchased from Peninsula Laboratories (Belmont, CA). Other chemicals used in this study were fura-2AM, phosbol 12-myristate 13-acetate (PMA), 1,2-bis(2-iminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (BAPTA-AM), pluronic acid F-127 (Molecular Probes, Eugene, OR), pertussis toxin (Sigma, St. Louis, MO), horse radish peroxidase-conjugated anti-phosphotyrosine (Upstate Biotechnology, Lake Placid, NY), genistein, herbimycin A (Life Technologies, Gaithersburg, MD), and monoclonal antibodies each direct against PLC-β3, -γ1, and -γ2 (Santa Cruz Biotechnology, Santa Cruz, CA). Santa Cruz indicates that the antibodies are specific for PLC-β3, -γ1, or -γ2 and do not cross-react with other isoforms.

3. Results

3.1. Presence of CRF-R2β in A-431 cells

We performed RT-PCR assays in RNA extracted from A-431 cells using CRF-R1, and -R2 specific primers to

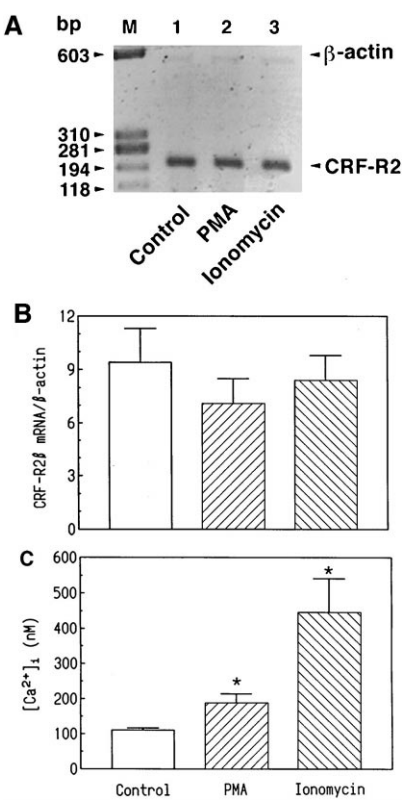

Fig. 2. No alteration of CRF-R2β mRNA in cells treated with PMA or ionomycin. Cells were treated with PMA (1 μM) or Ionomycin (1 μM) for 10 min. A representative gel of CRF-R2β mRNA expression is shown (A); the amount of expression was quantitated densitometrically (B); increases in $[Ca^{2+}]_i$ by PMA and ionomycin in fura-2AM-loaded cells were measured (C). Three independent experiments were conducted. Data are expressed as mean ± S.E.M. $* P < 0.05$ vs. respective control, Student's *t*-test.

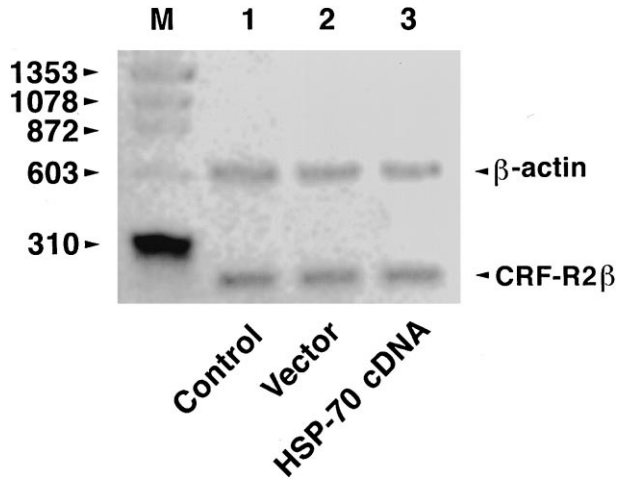

Fig. 3. Cells overexpressing HSP-70 do not display changes in CRF-R2β mRNA expression. Cells were transfected with HSP-70 cDNA 2 days prior to determining the levels of CRF-R2β mRNA expression. Three independent experiments were performed. A representative gel of CRF-R2β mRNA is shown.

determine which types of receptors are expressed. The appearance of a single band of 218 bp on agarose gels (Fig. 1A, lane 2) is consistent with the presence of CRF-R2 in these cells. No 300 bp band was detected that would indicate the presence of CRF-R1 mRNA (lane 1). Since two CRF-R2 isoforms are found in different tissues and organs, we used CRF-R2α and -R2β specific primers to determine which isoform is expressed. Fig. 1B shows that a PCR product 185 bp encoding CRF-R2β was detected (lane 2). No 300 bp band was detected that would indicate the presence of CRF-R2α mRNA (lane 1).

3.2. No alteration of CRF-R2β mRNA levels by activation of PKC or increases in $[Ca^{2+}]_i$

It is known that CRF acutely activates PKC-α and -β isoforms (Kiang et al., 1994) and increases $[Ca^{2+}]_i$ (Kiang, 1994, 1997). To determine whether the protein kinase C activator PMA or the Ca^{2+} ionophore ionomycin affected the amount of CRF-R2β, cells were treated with 1 μM PMA (10 min) or 1 μM ionomycin (10 min) prior to determining the levels of CRF-R2β mRNA. Neither PMA nor ionomycin altered the amount of CRF-R2β mRNA (Fig. 2A, lanes 2, 3 vs. lane 1, and Fig. 2B). However, as expected PMA and ionomycin increased resting $[Ca^{2+}]_i$ by 92% ± 14% and 266% ± 22%, respectively (Fig. 2C), which shows that the amount of CRF-R2β mRNA also is not regulated by Ca^{2+}.

3.3. Effect of HSP-70 overexpression on CRF-R2β

We reported previously that overexpression of HSP-70 cDNA in A-431 cells attenuates protein phosphorylation at serine residues (Ding et al., 1998) and desensitizes Ca^{2+}-associated machinery (Kiang, 1997; Kiang et al., 1998). We sought to measure CRF-R2β levels in cells overexpressing HSP-70, because it is known to be involved in Ca^{2+} signaling (Kiang, 1994, 1997). Using RT-PCR, both vector- and HSP-70 cDNA-transfected cells (Fig. 3, lane 2

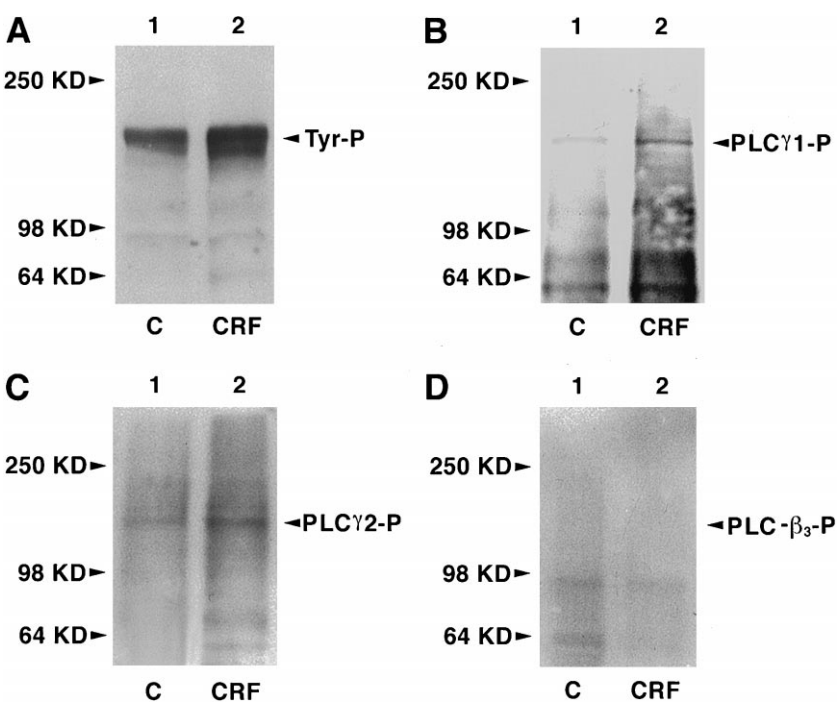

Fig. 4. Tyrosine phosphorylation of PLC-γ1 and -γ2 by CRF. Cells were treated with 1 nM CRF for 5 min. Twenty micrograms of the whole cell lysate was collected and blotted with the anti-phosphotyrosine antibody (A). In another experiment, 300 μg of the lysate was immunoprecipitated with each antibody directed to PLC-γ1 (B), -γ2 (C), or -β3 (D) prior to blotting with the anti-phosphotyrosine antibody. Three independent experiments were performed. Only increases in phosphorylation of PLC-γ1 and -γ2 by CRF at tyrosine residues were observed.

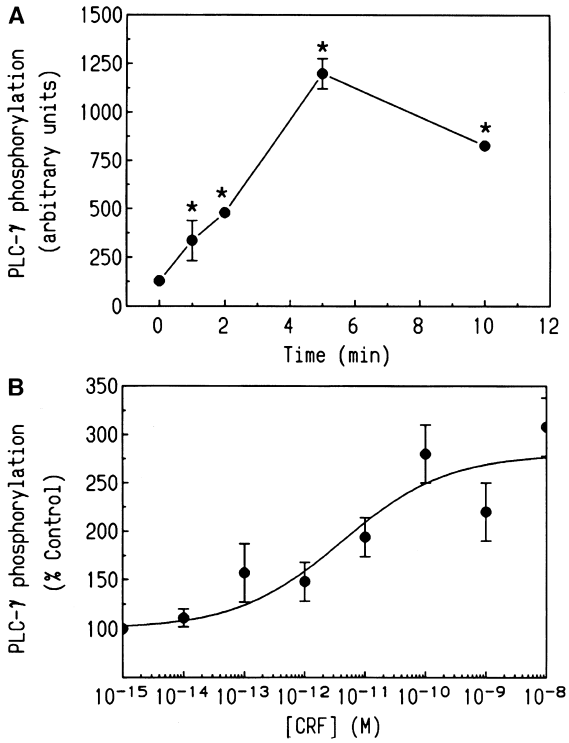

Fig. 5. CRF-induced PLC-γ phosphorylation. (A) Cells were treated with 1 nM CRF for 1, 2, 5, or 10 min. $* P < 0.05$ vs. time at 0 min, determined by one-way ANOVA and Studentized range-test. (B) Cells were treated with CRF at various concentrations for 5 min. The curve-fitted plot (using the Inplot program) of CRF concentrations vs. PLC-γ phosphorylation is used to calculate the median effective concentration (EC_{50}) of 4.2 ± 0.2 pM ($n = 3$–4 for each concentration).

vs. lane 3) expressed comparable amounts of CRF-R2β mRNA as determined densitometrically (in CRF-R2β/β-actin, control: 1.24 ± 0.1; vector: 1.25 ± 0.2; HSP-70 genetransfection: 1.28 ± 0.1, $P > 0.05$, one-way ANOVA).

3.4. Tyrosine phosphorylation of PLC-γ by CRF

Previously this laboratory reported that 10 min treatment of cells with CRF increased protein tyrosine phosphorylation at molecular weight of approximate 150 kDa (Kiang, 1997). To determine the identity of this phosphorylated protein (Fig. 4A), the cell lysate was immunoprecipitated using monoclonal antibodies directed against each PLC-β3, -γ1, and -γ2 isoform and blotted with anti-phosphotyrosine antibody. We used these three antibodies because treatment with CRF can increase cellular levels of inositol 1,4,5-trisphosphate (Kiang, 1994), which is a product resulting from PLC activation, and because PLC-β3, -γ1, and -γ2 have their molecular weights of approximate 150 kDa. Since these monoclonal antibodies against each PLC-β3, -γ1, and -γ2 isoform are specific and do not cross-react with other isoforms, the blotted immunoprecipitates indicate the presence of PLC-γ1 (Fig. 4B, lane 1) and -γ2 (Fig. 4C, lane 1), but not -β3 (Fig. 4D). Treatment of cells with CRF increased phosphorylation of PLC-γ1 (Fig. 4B, lane 2) and PLC-γ2 (Fig. 4C, lane 2) at tyrosine residues. Therefore, in subsequent experiments, immunoprecipitation of cell lysates directed against PLC-γ1 and -γ2 and Western blots with anti-phosphotyrosine antibody were performed.

The increase in PLC-γ phosphorylation by CRF occurred in a time- and concentration-dependent fashion. Cells treated with 1 nM CRF for 1, 2, 5, or 10 min displayed maximal phosphorylation of PLC-γ by 5 min, which remained above the basal level even after 10 min (Fig. 5A). The median effective concentration (EC_{50}) of CRF was 4.2 ± 0.1 pM (Fig. 5B).

3.5. Inhibition of the CRF-induced PLC-γ phosphorylation by a CRF antagonist but not pertussis toxin

To determine whether elevated PLC-γ phosphorylation was a CRF receptor-mediated process, cells were pretreated with 50 nM of CRF receptor antagonist α-helical CRF (9–41) for 1 min prior to treatment with CRF. Fig. 6 shows that the elevation of protein phosphorylation was inhibited by treatment with α-helical CRF (9–41), suggesting that the CRF-induced increase in PLC-γ phosphorylation is mediated by the CRF-R2β.

Previously, this laboratory reported that the CRF-induced increase in $[Ca^{2+}]_i$ is mediated by G-proteins coupled CRF receptors because it was partially inhibited following prolonged treatment of cells with pertussis toxin, a known G_i inhibitor (Kiang, 1994). The remaining portion of the CRF-induced increase in $[Ca^{2+}]_i$ must be mediated by other unknown mechanisms. Therefore, we sought to determine whether pertussis toxin sensitive G-proteins were involved in the PLC-γ phosphorylation. Cells were treated with pertussis toxin (30 ng ml^{-1}, 24 h) prior to CRF at 1 nM for 5 min. Pertussis toxin alone neither altered the

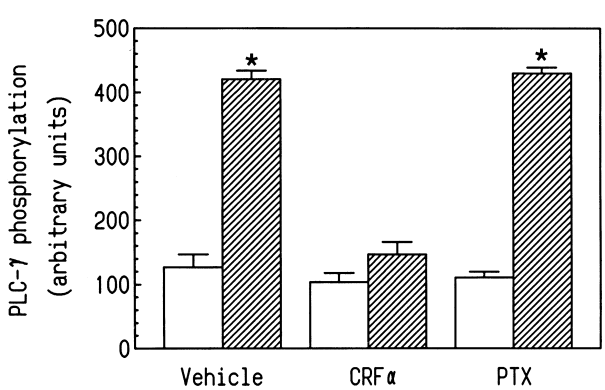

Fig. 6. Inhibition of the CRF-induced PLC-γ phosphorylation by a CRF antagonist. Cells were treated with CRF antagonist (CRFα; 50 nM, 1 min) or pertussis toxin (PTX; 30 ng ml^{-1}, 24 h) prior to 1 nM CRF for 5 min. Three independent experiments were conducted. $* P < 0.05$ vs. controls, determined by two-way ANOVA and Studentized range-test. □: controls; square with left-slanted lines: CRF-treated.

basal nor inhibited the CRF-induced levels of the PLC-γ phosphorylation (Fig. 6). This observation suggests that the CRF-induced PLC-γ tyrosine phosphorylation is mediated through CRF-R2β that is not coupled to pertussis toxin-sensitive G proteins.

3.6. Inhibition of the CRF-induced PLC-γ phosphorylation by inhibitors of protein tyrosine kinases

To determine whether the CRF-induced PLC-γ phosphorylation is mediated by activation of protein tyrosine kinases, cells were treated with protein tyrosine kinase inhibitors, genistein (100 μM, 10 min) or herbimycin A (1 μg ml^{-1}, 30 min), prior to CRF challenge. Treatment of cells with genistein suppressed the CRF-induced PLC-γ phosphorylation to levels even lower than the baseline. Treatment with herbimycin A also attenuated the phosphorylation statistically significantly albeit partially. Both herbimycin A and genistein alone did not change the basal level of PLC-γ phosphorylation (Fig. 7). The results suggest that the CRF-induced PLC-γ phosphorylation is activated by protein tyrosine kinases.

3.7. Ca^{2+}-dependence of the CRF-induced PLC-γ phosphorylation

Because CRF increases $[Ca^{2+}]_i$ by increasing the Ca^{2+} entry through CRF receptor-operated Ca^{2+} channels and Ca^{2+} mobilization from the inositol 1,4,5-trisphosphate-sensitive Ca^{2+} pool (Kiang, 1997) and because removal of external Ca^{2+} blocks this increase, the association of the CRF-induced PLC-γ phosphorylation with $[Ca^{2+}]_i$ was determined. Cells were treated with 100 μM BAPTA-AM for 15 min prior to treatment with CRF. Fig. 8 shows that BAPTA-AM chelated cytosolic free Ca^{2+} and attenuated the CRF-induced PLC-γ phosphorylation, suggesting that activation of PTK depended on $[Ca^{2+}]_i$.

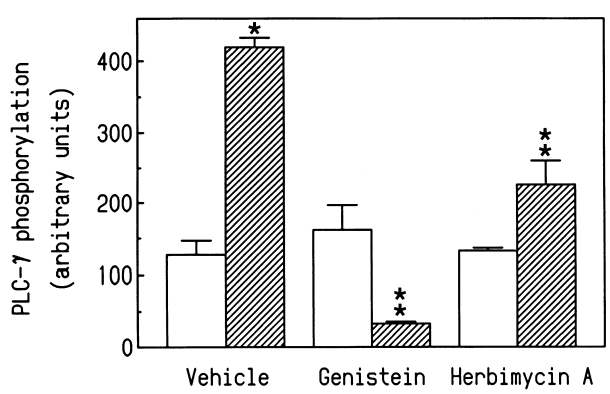

Fig. 7. Inhibition of the CRF-induced PLC-γ phosphorylation by protein tyrosine kinase inhibitors. Cells were treated with genistein (100 μM, 10 min) or herbimycin A (1 μg ml^{-1}, 30 min) prior to 1 nM CRF for 5 min. Three independent experiments were conducted. $* P < 0.05$ vs. controls; $** P < 0.05$ vs. vehicle control, vehicle CRF-treated, genistein control, and herbimycin A control, determined by two-way ANOVA and Studentized range-test. □: controls; square with left-slanted lines: CRF-treated.

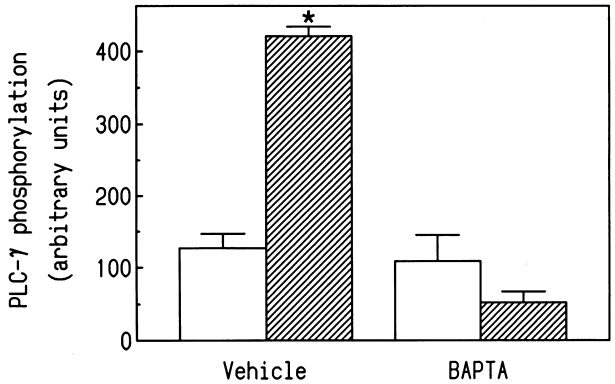

Fig. 8. Ca^{2+}-dependence of the CRF-induced PLC-γ phosphorylation. Cells were treated with BAPTA-AM (100 μM, 15 min) prior to CRF (1 nM, 5 min). Three independent experiments were conducted. $* P < 0.05$ vs. controls, determined by Student's *t*-test. □: controls; square with left-slanted lines: CRF-treated.

4. Discussion

This study demonstrates that A-431 cells express CRF-R2β but not -R1 or -R2α mRNA. The levels of its expression are not altered by 10 min treatment with protein kinase C activator, Ca^{2+} ionophore, or overexpression of HSP-70, although treatment of cells with CRF immediately increases $[Ca^{2+}]_i$ (Kiang, 1994, 1997). CRF receptors of two types have been found in a variety of cells and organs. CRF-R1 mRNA is predominantly expressed in cerebellum, pituitary, cerebral cortex, olfactory bulb, and Leydig cells (see review, De Souza, 1992; Potter et al., 1994; Heinrich et al., 1998). There have been two CRF-R2 subtypes described, α and β. CRF-R2α mRNA is found exclusively in hypothalamus, lateral septum, and olfactory bulb, whereas mRNA from CRF-R2β is expressed elsewhere in the brain as well as in lung, heart, and skeletal muscle (Kishimoto et al., 1995; Lovenberg et al., 1995b; Perrin et al., 1995). CRF-R2β was observed in A-431 cells, a skin cell line.

It has been shown in rat anterior pituitary cell culture that treatment with endotoxin or interleukin-1β reduces CRF-R1 mRNA by 57 and 47%, respectively, whereas prolonged exposure of pituitary to CRF or vasopressin does not alter CRF-R1 mRNA expression (Aubry et al., 1997). The regulation of the expression of CRF-R2β mRNA has not been studied so far. In A-431 cells, CRF-R2β mRNA expression was not altered by 10 min treatment with PMA or ionomycin, suggesting that CRF-R2β expression is independent of protein kinase C activation and increases in $[Ca^{2+}]_i$. Our earlier data had indicated that cells overexpressing HSP-70 displayed increases in the resting $[Ca^{2+}]_i$ (Kiang et al., 1998) and decreases in protein phosphorylation (Ding et al., 1998). Nevertheless,

CRF-R2β mRNA expression did not change even after elevation of HSP-70 levels by HSP-70 cDNA-transfection. HSP-70 overexpression does not alter $[Ca^{2+}]_i$ responding to CRF (Kiang et al., 1996a).

Ten mammalian isoforms of PLC have been identified to date, and are grouped into β type (PLC-β1, 2, 3, and 4), γ type (PLC-γ1 and 2), and δ-type (PLC-δ1, 2, 3, and 4). The distinct structural features of the different PLC types have been associated with specific mechanisms of receptor-mediated enzyme activation. PLC-β isoforms are activated by heterotrimeric G proteins; PLC-γ isoforms are activated by tyrosine phosphorylation; the mechanisms which lead to the activation of PLC-δ isoforms are unclear (Cockcroft and Thomas, 1992; Noh et al., 1995). Phosphorylation of PLC-γ is known to increase inositol 1,4,5-trisphosphate and diacylglycerol. The former mobilizes Ca^{2+} from intracellular pools and the latter activates protein kinase C (Lee and Rhee, 1995). In human epidermoid A-431 cells, PLC-γ1 and -γ2 but not -β3 are detected.

Our data indicate that CRF binds to CRF-R2β, activates protein tyrosine kinases, which phosphorylates PLC-γ isoforms at tyrosine residues. Treatment of cells with a CRF receptor antagonist (Fig. 6) or inhibitors of protein tyrosine kinases (Fig. 7) suppressed the CRF-induced PLC-γ tyrosine phosphorylation (Fig. 7). Our preliminary data indicate that tyrosine phosphorylation of PLC-γ isoforms represents the PLC-γ activation because protein tyrosine kinase inhibitors are capable of inhibiting the CRF-induced increase in inositol 1,4,5-trisphosphate. It is not known whether the CRF-R2β has intrinsic protein tyrosine kinase activity similar to that expressed by the receptors for epidermal growth factor, fibroblast growth factor, nerve growth factor, platelet-derived growth factor, monocyte colony stimulating factor, stem cell factor, and insulin (Jerome et al., 1991). Unlike PLC-β and -δ isoforms, PLC-γ isoforms contain a Src homology 3 (SH3) domain, which is characterized by the ability to bind proline-rich sequences such as PPLPXR and RXLPPXP (critical prolines are underlined; X indicates any amino acid; other residues are partially conserved; see reference by Feng et al., 1994). The CRF-R2β contains 24 prolines in a whole length of 431 amino acid residues with two proline-rich regions: PSGPPG and CLLPPPL. Therefore, it is highly likely that CRF-R2β may intrinsically facilitate the binding and phosphorylation of PLC-γ isoforms after it is activated by CRF. The experiments to determine whether CRF-R2β may possess intrinsic tyrosine kinase activity are undergoing in our laboratory.

Stimulation of non-receptor protein tyrosine kinases is known to phosphorylate and activate PLC-γ1 in rat aortic smooth muscle cells (Marrero et al., 1995). Liao et al. (1993) reported in vitro tyrosine phosphorylation of PLC-γ1 and -γ2 by src-family protein tyrosine kinases, including p561ck, p53/56lyn, p59hck, p59fyn, and p60src, in response to the activation of cell surface receptors. Therefore, the possibility of CRF activating the src-family protein tyrosine kinases via CRF-R2β cannot be excluded either.

The CRF induced tyrosine phosphorylation of PLC-γ was determined by immunoprecipitation and Western blotting. There are additional protein bands present at lower molecular weights on the Western blots (Fig. 4). We assume the appearance of these bands is nonspecific, even though the antibodies used for the study were specific. However, these results do not rule out completely the possibility that CRF induces tyrosine phosphorylation of proteins other than PLC-γ.

The CRF-induced phosphorylation of PLC-γ was not altered by treatment of pertussis toxin. This observation is consistent with findings of the CRF-induced increase in $[Ca^{2+}]_i$ which treatment with pertussis toxin inhibits only partially. Therefore, the CRF-induced increase in $[Ca^{2+}]_i$ is partly contributed by PLC-γ tyrosine phosphorylation which is not coupled to G proteins.

Despite of independence of G proteins, the CRF-induced PLC-γ tyrosine phosphorylation is regulated by the level of $[Ca^{2+}]_i$. Treatment of cells with CRF induces maximal increase in $[Ca^{2+}]_i$ within seconds with an EC_{50} of 6.8 ± 0.1 pM (Kiang, 1994). On the contrary, the increase in PLC-γ phosphorylation increases slowly and reaches the maximum by 5 min with an EC_{50} of 4.2 ± 0.1 pM, suggesting that the PLC-γ phosphorylation is secondary to the increase in $[Ca^{2+}]_i$. The observation that BAPTA chelates cytosolic free Ca^{2+} blocks the CRF-induced PLC-γ phosphorylation further supports the view. Similar finding was observed with $cPLA_2$ (Nalefski et al., 1994; Glover et al., 1995; Schievella et al., 1995).

Data presented in this study and previous studies (Kiang, 1994, 1997) suggest the existence of a mechanism whereby the phosphorylation of PLC-γ induced by CRF to increase inositol 1,4,5-trisphosphate. CRF binds to CRF receptor type 2β, which activates PLC-β isoforms and phosphorylates PLC-γ isoforms and then leads to increased levels of inositol trisphosphate and protein kinase C activity. The increase in inositol 1,4,5-trisphosphate mobilizes Ca^{2+} from inositol 1,4,5-trisphosphate-sensitive intracellular Ca^{2+} stores.

In summary, CRF-R2β is present in human epidermoid A-431 cells and the levels of its message are not altered by protein kinase C activation, increases in $[Ca^{2+}]_i$, or overexpression of HSP-70. CRF induced phosphorylation of PLC-γ1 and -γ2 isoforms at tyrosine residues via CRF-R2β and activation of protein tyrosine kinases with a value of EC_{50} of 4.2 ± 0.1 pM. The phosphorylation was not mediated by pertussis toxin-sensitive G proteins but depended on the level of $[Ca^{2+}]_i$.

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References

Aubry, J.-M., Turnbull, A.V., Pozzoli, G., Rivier, C., Vale, W., 1997. Endotoxin decreases corticotropin-releasing factor receptor 1 messenger ribonucleic acid levels in the rat pituitary. Endocrinology 138, 1621–1626.

Chen, R., Lewis, K.A., Perrin, M.H., Vale, W.W., 1993. Expression cloning of a human corticotropin-releasing factor receptor. Proc. Natl. Acad. Sci. USA 90, 8967–8971.

Cockcroft, S., Thomas, G.M.H., 1992. Inositol–lipid-specific phospholipase C isoenzymes and their differential regulation by receptors. Biochem. J. 288, 1–14.

De Souza, E.B., 1992. Corticotropin-releasing hormone receptors. In: Bjorklund, A., Hokfelt, T., Kuhar, M.J. (Eds.), Handbook of Chemical Neuroanatomy—Neuropeptide Receptors in the CNS, Part III. Elsevier, Amsterdam, pp. 145–185.

Ding, S.Z., Tsokos, G.C., Kiang, J.G., 1998. Overexpression of HSP-70 inhibits the phosphorylation of HSF1 by activating protein phosphatase and inhibiting protein kinase C activity. FASEB J. 12, 451–459.

Feng, S., Chen, J.K., Yu, H., Simon, J.A., Schreiber, S.L., 1994. Two binding orientations for peptides to the src SH_3 domain: development of a general model for SH_3-ligand interactions. Science 266, 1241–1247.

Glover, S., de Carvalho, M.S., Bayburt, T., Jonas, M., Chi, E., Leslie, C.C., Gelb, M.H., 1995. Translocation of the 85-kDa phospholipase A_2 from cytosol to the nuclear envelope in rat basophilic leukemia cells stimulated with calcium ionophore or IgE/antigen. J. Biol. Chem. 270, 15359–15367.

Heinrich, J., Meyer, M.R., Furkert, J., Sasse, A., Beyermann, M., Bonigk, W., Berger, H., 1998. Corticotropin-releasing factor (CRF) agonists stimulate testosterone production in mouse Leydig cells through CRF receptor-1. Endocrinology 139, 651–658.

Hunt, C., Morimoto, R.I., 1985. Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. Proc. Natl. Acad. Sci. USA 82, 6455–6459.

Jerome, V., Leger, J., Devin, J., Baulieu, E.E., Catelli, M.G., 1991. Growth factors acting via tyrosine kinase receptors induce HSP-90 alpha gene expression. Growth Factors 4, 317–320.

Kiang, J.G., 1994. Corticotropin-releasing factor increases cytosolic free calcium via receptor-mediated Ca^{2+} channels. Eur. J. Pharmacol. (Molecular Pharmacol. Section) 267, 135–142.

Kiang, J.G., 1997. Corticotropin-releasing factor-like peptides increase cytosolic free calcium in human epidermoid A-431 cells. Eur. J. Pharmacol. 329, 237–244.

Kiang, J.G., Wei, E.T., 1985. CRF: an inhibitor of neurogenic plasma extravasation produced by saphenous nerve stimulation. Eur. J. Pharmacol. 114, 111–112.

Kiang, J.G., Wei, E.T., 1987. Corticotropin-releasing factor (CRF) inhibits thermal injury. J. Pharmacol. Exp. Ther. 243, 517–520.

Kiang, J.G., Wang, X.D., McClain, D.E., 1994. Corticotropin-releasing factor increases protein kinase C activity by elevating membrane-bound α and β isoforms. Chin. J. Physiol. 37, 105–110.

Kiang, J.G., Ding, X.Z., McClain, D.E., 1996a. Thermotolerance attenuates heat-induced increases in $[Ca^{2+}]_i$ and HSP-72 synthesis but not heat-induced intracellular acidification in human A-431 cells. J. Invest. Med. 44, 189–192.

Kiang, J.G., Wang, X.D., Ding, X.Z., Gist, I.D., Smallridge, R.C., 1996b. Heat shock inhibits the hypoxia-induced effects on iodide uptake and signal transduction and enhances cell survival in rat thyroid FRTL-5 cells. Thyroid 6, 475–483.

Kiang, J.G., Ding, X.Z., McClain, D.E., 1998. Overexpression of HSP-70 attenuates increases in $[Ca^{2+}]_i$ and protects human epidermoid A-431 cells after chemical hypoxia. Toxicol. Appl. Pharmacol. 149, 185–194.

Kishimoto, T., Pearse, R.V., Lin, C.R., Rosenfeld, M.G., 1995. A sauvagine/corticotropin-releasing factor receptor expressed in heart and skeletal muscle. Proc. Natl. Acad. Sci. USA 92, 1108–1112.

Lee, S.B., Rhee, S.G., 1995. Significance of PIP_2 hydrolysis and regulation of phospholipase C isozymes. Curr. Opin. Cell. Biol. 7, 183–189.

Liao, F., Shin, H.S., Rhee, S.G., 1993. In vitro tyrosine phosphorylation of PLC-γ1 and PLC-γ2 by src-family protein tyrosine kinases. Biochem. Biophys. Res. Commun. 191, 1028–1033.

Lovenberg, T.W., Liaw, C.W., Grigoriadis, D.E., Clevenger, W., Chalmers, T., De Souza, E.B., Oltersorf, T., 1995a. Cloning and characterization of a functionally distinct corticotropin-releasing factor subtype from rat brain. Proc. Natl. Acad. Sci. USA 92, 836–840.

Lovenberg, T.W., Chalmers, D.T., Liu, C., De Souza, E.B., 1995b. CRF2α and CRF2β receptor mRNAs are differentially distributed between the rat central nervous system and peripheral tissues. Endocrinology 136, 4139–4142.

Marrero, M.B., Schieffer, B., Paxton, G., Shieffer, E., Bernstein, K.E., 1995. Electroporation of pp60c-src antibodies inhibits the angiotensin II activation of phospholipase C-γ1 in rat aortic smooth muscle cells. J. Biol. Chem. 270, 15734–15738.

Nalefski, E.A., Sultzman, L.A., Martin, D.M., Kriz, R.W., Towler, P.S., Knopf, J.L., Clark, J.D., 1994. Delineation of two functionally distinct domains of cytosolic phospholipase A_2, a regulatory Ca^{2+}-dependent lipid-binding domain and a Ca^{2+}-independent catalytic domain. J. Biol. Chem. 269, 18239–18249.

Noh, D.-Y., Shin, S.H., Rhee, S.G., 1995. Phosphoinositide-specific phospholipase C and mitogenic signaling. Biochim. Biophys. Acta 1242, 99–114.

Perrin, M.H., Donaldson, C.J., Chen, R., Lewis, K.A., Vale, W.W., 1993. Cloning and functional expression of a rat brain corticotropin-releasing factor (CRF) receptor. Endocrinology 133, 3058–3061.

Perrin, M., Donaldson, C., Chen, R., Blount, A., Berggtren, T., Bilezikjian, L., Sawchenko, P., Vale, W., 1995. Identification of a second CRF receptor gene and characterization of a cDNA expressed in heart. Proc. Natl. Acad. Sci. USA 92, 2926–2973.

Potter, E., Sutton, S., Donaldson, C., Chen, R., Perrin, M., Lewis, K., Sawchenko, P.E., Vale, W., 1994. Distribution of corticotropin-releasing factor receptor expression in the rat brain and pituitary. Proc. Natl. Acad. Sci. USA 91, 8777–8781.

Schievella, A.R., Reigier, M.K., Smith, W.L., Lin, L.L., 1995. Calcium-mediated translocation of cytosolic phospholipase A_2 to the nuclear envelope and endoplasmic reticulum. J. Biol. Chem. 270, 30749–30754.

Wei, E.T., Kiang, J.G., 1987. Inhibition of protein exudation from the trachea by corticotropin-releasing factor. Eur. J. Pharmacol. 140, 63–67.