Mediation of Corticotropin Releasing Factor Type 1 Receptor Phosphorylation and Desensitization by Protein Kinase C: A Possible Role in Stress Adaptation

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ABSTRACT

Protein kinase C (PKC)-mediated desensitization of the corticotropin releasing factor type 1 (CRF1) receptor was investigated in human retinoblastoma Y79 and transfected COS-7 cells. Because stimulation of Y79 cells with CRF resulted in large (∼30-fold) increases in intracellular cAMP accumulation without changing inositol phosphate levels, the CRF1 receptor expressed in retinoblastoma cells couples to Gs, but not to Gi, and predominantly signals via the protein kinase A cascade. Direct activation of PKC by treatment with the phorbol ester phorbol 12-myristate 13-acetate (PMA) or 1,2-dioctanoyl-sn-glycerol (DOG) desensitized CRF1 receptors in Y79 cells, reducing the maximum for CRF- (but not forskolin)-stimulated cAMP accumulation by 56.3 ± 1.2% and 40.4 ± 2.1%, respectively (p < 0.001). Pretreating Y79 cells with the PKC inhibitor bisindolylmaleimide I (BIM) markedly inhibited PMA’s desensitizing action on CRF-stimulated cAMP accumulation, but did not affect homologous CRF1 receptor desensitization. Retinoblastoma cells were found to express PKCα, β1, βII, δ, λ, and RACK1. When α and β isoforms of PKC were down-regulated 80 to 90% by a 48-h PMA exposure, PMA-induced CRF1 receptor desensitization was abolished. In transfected COS-7 cells the magnitude of CRF1 receptor phosphorylation after a 5-min exposure to PMA was 2.32 ± 0.21-fold greater compared with the basal level. Pretreating COS-7 cells with BIM abolished PMA-induced CRF1 receptor phosphorylation. These studies demonstrate that protein kinase C (possibly α and β isoforms) has an important role in the phosphorylation and heterologous desensitization of the CRF1 receptor.

Cellular signaling mediated by agonist-induced G protein-coupled receptor (GPCR) activation must be stringently regulated to prevent an unrestrained level of receptor stimulation. Two major modes of terminating GPCR signaling have been identified as homologous and heterologous desensitization. Homologous desensitization refers to an agonist-dependent mechanism of cellular adaptation whereby signal transduction becomes rapidly terminated in the continued presence of high agonist concentrations. This begins with the selective translocation of a G protein-coupled receptor kinase (GRK) from the cytosol to the membrane, where it phosphorylates specific serine and threonine residues in the GPCR’s C-terminal tail and/or third intracellular loop (Penn and Benovic, 1998; Ciang et al., 2002; Pierce et al., 2002; Kohout and Lefkowitz, 2003).

The neuropeptide CRF regulates behavioral, neuroendocrine, and autonomic responses to stress by acting at two high-affinity CRF receptors (CRF1 and CRF2) in the amygdala and its extended neurocircuits, as well as in adrenocorticotropic-secreting pituitary corticotropes (Grigoriadis et al., 2001; Dautzenberg and Hauger, 2002; Perrin and Vale, 2002). CRF1 receptors are rapidly desensitized in retinoblastoma cells. Because stimulation of Y79 cells with CRF resulted in large (∼30-fold) increases in intracellular cAMP accumulation without changing inositol phosphate levels, the CRF1 receptor expressed in retinoblastoma cells couples to Gs, but not to Gi, and predominantly signals via the protein kinase A cascade. Direct activation of PKC by treatment with the phorbol ester phorbol 12-myristate 13-acetate (PMA) or 1,2-dioctanoyl-sn-glycerol (DOG) desensitized CRF1 receptors in Y79 cells, reducing the maximum for CRF- (but not forskolin)-stimulated cAMP accumulation by 56.3 ± 1.2% and 40.4 ± 2.1%, respectively (p < 0.001). Pretreating Y79 cells with the PKC inhibitor bisindolylmaleimide I (BIM) markedly inhibited PMA’s desensitizing action on CRF-stimulated cAMP accumulation, but did not affect homologous CRF1 receptor desensitization. Retinoblastoma cells were found to express PKCα, β1, βII, δ, λ, and RACK1. When α and β isoforms of PKC were down-regulated 80 to 90% by a 48-h PMA exposure, PMA-induced CRF1 receptor desensitization was abolished. In transfected COS-7 cells the magnitude of CRF1 receptor phosphorylation after a 5-min exposure to PMA was 2.32 ± 0.21-fold greater compared with the basal level. Pretreating COS-7 cells with BIM abolished PMA-induced CRF1 receptor phosphorylation. These studies demonstrate that protein kinase C (possibly α and β isoforms) has an important role in the phosphorylation and heterologous desensitization of the CRF1 receptor.
PKC Phosphorylates and Desensitizes CRF1 Receptors

Materials and Methods

Materials. Reagent purchases were as follows. 1) Bovine serum albumin (BSA, fraction V), isobutylmethylxanthine, forskolin, and other highly pure chemicals: Sigma-Aldrich (St. Louis, MO); 2) aprotonin (Trasylol), phorbol 12-myristate 13-acetate (PMA), 4a-phorbol 12-myristate 13-acetate (an inactive PMA isomer), 1,2-dioctanoyl-sn-glycerol (DOG), bisindolylmaleimide I (BIM), staurosporine: Calbiochem (San Diego, CA); 3) defined fetal bovine serum (SH30070.03): Hyclone Laboratories (Logan, UT). The University of California, San Diego Cell Culture Core Facility supplied all other cell culture reagents (Mediatech, Herndon, VA). Ovine CRF (CRF: Bachem California, Torrance, CA; purity >98%) was used to stimulate cAMP accumulation in all experiments and to desensitize CRF1 receptors.

All SDS-PAGE reagents were purchased from Invitrogen-Novex (Carlsbad, CA). For CRF1 receptor phosphorylation experiments, the following reagents were used: 1) protein A-Sepharose (PRA-Seph) (Oncogene Research Products, San Diego, CA); 2) HA.11 mouse monoclonal anti-HA antibody (Boehringer, Richmond, CA).

Cell Culture and Transfection. Suspension human retinoblastoma Y79 cells were grown at a density of 5 to 8×10^6 cells/flask in RPMI 1640 and used between passages 4 and 25 as previously described (Hauger et al., 1997; Dautzenberg et al., 2001a). COS-7 cells were seeded at 6×10^5 cells/10-cm dish in DMEM containing 10% (v/v) fetal bovine serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin (COS-7 cell medium). COS-7 cells were cultured for 3 days before transfection using 5 ml of OptiMEM containing 10 μg/ml LipofectAMINE (Invitrogen, Carlsbad, CA) and 5 μg of HA-tagged CRF1 receptor cDNA for 6 h at 37°C as described (Hauger et al., 2000). After changing to fresh COS-7 cell medium, the cells were cultured for a further 2 days before use.

Second Messenger Assays. Following extensive cell washing, intracellular cAMP levels were measured in ether-extracted and acetylated cell lysates using a double-antibody radioimmunoassay kit (cAMP [125I] assay system, RPA 509; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK), as previously described (Hauger et al., 1997; Dautzenberg et al., 2001a). For inositol phosphate (IP) experiments, Y79 cells were metabolically labeled with myo-[2-3H]inositol (5 μCi/ml) overnight (18 h). After labeled cells were washed twice with a large volume (40 ml) of myoinositol-free RPMI 1640, they were preincubated in myoinositol-free Medium 199/E with 10 mM lithium chloride for 30 min at 37°C. After cells were again washed, centrifuged, and resuspended in myoinositol-free Medium 199/E with 10 mM lithium chloride, they were maximally stimulated with 1 μM CRF for 20 min. After the reaction was stopped by adding 10 mM formic acid, formation of IP_2 and IP_3 was measured by anion exchange chromatography using Bio-Rad AG 1-X8 columns, as previously described (Olivares-Reyes et al., 2001).

Western Blot Quantitation of PKC Protein Expression. Lysates of Y79 cells (20–30 μg per lane) were loaded onto 4 to 12% Tris-glycine gradient gels (Invitrogen-Novex) and proteins were resolved in a NOVEX Xcell II Mini-Cell System using SDS-PAGE under reducing conditions (Invitrogen-Novex Tris-glycine SDS sample buffer containing 5.0% β-mercaptoethanol) at a fixed 125 V (current 35–40 amps) for 90 min according to the method of Laemmli (Dautzenberg et al., 2001a; Dautzenberg and Hauger, 2001). After...
Western transfer of resolved retinoblastoma proteins onto polyvinylidene difluoride membranes (Invitrogen-Novex) was completed (Dautzenberg et al., 2001a; Dautzenberg and Hauger, 2001), blots were blocked for 1 h in a solution of Tris-buffered saline (20 mM Tris pH 7.5, 500 mM NaCl) with 0.2% Tween 20 and 4% BSA (TTBS-BSA) with constant shaking at room temperature. Blots were then washed with TTBS-BSA and immunoprobed overnight (~18 h) at 4°C with constant shaking with one of the following antibodies: 1) a mouse monoclonal antibody targeting PKCs (610108; 1:1,000), PKCα (611158; 1:1,000), PKCδ (610397; 1:500), PKCε (610085; 1:1,000), PKCa (610814; 1:250), PKCa (610089; 1:250), PKCi (610175; 1:250), PKCδ (610207; 1:250), RACK1 (610178; 1:2,500), or DGKα (610930; 1:250) (BD Biosciences Pharmingen Transduction Laboratory; San Diego, CA); 2) a mouse monoclonal targeting PKCβ1 (E-3/3-6-049; 1:100) or a rabbit polyclonal targeting PKCβII (C-18/sc-210; 1:200) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); or 3) a rabbit polyclonal antibody (P500; 1:3,000, kindly provided by Dr. A. Newton, Department of Pharmacology, University of California, San Diego) targeting the phosphorylated activation loop of protein kinase CβII (Dutil and Newton, 2000). After the membranes were washed in TTBS-BSA (six 10-min washes), blots were incubated for 1 h at room temperature with constant shaking with one of the following antibodies (in TTBS-BSA): 1) sheep anti-mouse IgG-HRP (NA931, 1:5,000; Amersham Biosciences Inc., Piscataway, NJ) or 2) donkey anti-rabbit IgG-HRP (NA934, 1:5,000; Amersham Biosciences Inc.). After membranes were washed extensively in TTBS (six 20-min washes), chemiluminescence detection of Western blots was performed using ECL Plus (Amersham Biosciences Inc.).

CRF, Receptor Phosphorylation Assay. Phosphorylation of the CRF1 receptor was determined as previously described (Hauger et al., 2001a). Briefly, transfected COS-7 cells in 10-cm dishes were solubilized in LB Plus (Amersham Biosciences Inc.) at the end of the pretreatment period, basal cAMP levels were metabolically labeled for 4 h at 37°C in 5 ml of P-free DMEM containing 0.1% (w/v) BSA and 100 μCi/ml 32P. Cells were then pretreated with vehicle or BIM for 30 min followed by a 15-min exposure to PMA for 5 min. Treated cells were lysed in buffer (LB: 50 mM Tris, pH 8.0, 100 mM NaCl, 20 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 10 mg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml pepstatin, 10 μg/ml benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 μM okadaic acid) and probe-sonicated twice for 20 s. After removal of nuclei at 750g, membranes were pre-extracted by the addition of an equal volume of LB containing 2 M NaCl and 8 M urea followed by overnight tumbbling at 4°C. The membranes were then collected at 200,000g and solubilized in LB + [LB supplemented with 1% Triton X-100, 10 mM β-glycerolphosphate, and 0.1% (w/v) SDS] with Dounce homogenization. After clarification at 14,000g, solubilized membranes were preclared by being incubated with 2% (w/v) protein A-Sepharose for 1 h at 4°C. Immunoprecipitation of CRF1 receptors was performed by adding 1 μl of HA.11 antibody and 2% (w/v) protein A-Sepharose and incubating overnight at 4°C. After washing of the Sepharose-bound immune complexes in LB + lacking protease inhibitors, 32P-labeled phospho-HA-CRF1 receptors were eluted in Laemmli sample buffer for 1 h at 48°C and resolved by SDS-PAGE (8–16% gradient resolving gel) before visualization in a PhosphorImager (Amersham Biosciences Inc.).

Data Reduction and Statistical Analyses. CRF1, receptor desensitization data were calculated as percentage of control values as previously described (Hauger et al., 1997). Data reduction for the cyclic AMP radioimmunoassay was performed using a log-log program. Analyses of variance (ANOVA) across experimental groups were performed on a MacIntosh PC using PRISM Version 2.0 (GraphPad Software Inc., San Diego, CA). If the one-way ANOVA was statistically significant, planned post hoc analyses were performed using Bonferroni’s multiple comparison tests to determine individual group differences. Immunoreactive PKC protein bands on Western blots were quantitated and analyzed on the STORM imager using ImageQuant software (Amersham Biosciences Inc.) (Dautzenberg et al., 2001a).

Results

Time Course for PMA-Induced CRF1, Receptor Desensitization in Y79 Cells. We began our study by investigating potential PKC regulation of CRF1 receptor signaling in human retinoblastoma Y79 cells. Although PMA-induced PKC activation augments the ability of CRF to stimulate cAMP accumulation and adrenocorticotropin release in anterior pituitary cells (Abou-Samra et al., 1987), the magnitude of cAMP accumulation stimulated by 100 nM CRF was decreased by ~50% when Y79 cells were coincubated with 100 nM PMA during the 15-min stimulation period (Fig. 1A). When Y79 cells were stimulated with 1 μM CRF for 20 min, inositol phosphate levels were not increased above basal (i.e., no CRF stimulation) values (Fig. 1B) in contrast to a 29.5 ± 0.9-fold increase in cAMP formation observed in CRF-stimulated cells (p < 0.0001; data not shown). Previous studies have shown that PKC participates in both homologous and heterologous GPCR desensitization by promoting the phosphorylation of serines and threonines within consensus sites located in a receptor’s C terminus and/or other intracellular loops (Penn and Benovic, 1998; Pierce et al., 2002). When Y79 cells were pretreated for 1 h with 1 μM PMA to maximally activate PKC, the magnitude of cAMP accumulation following subsequent restimulation with 100 nM CRF was decreased by ~50% (p < 0.0001) (Fig. 1C), thereby providing evidence for PKC mediation of CRF1 receptor desensitization. When the reversibility of PMA-induced desensitization of CRF1 receptors was investigated, we found that CRF-stimulated cAMP stimulation failed to recover from the desensitized state in Y79 cells that were exposed to 1 μM PMA for 1 h, washed extensively, and then cultured for an additional 4 h (data not shown). The specificity of PMA-induced CRF1, receptor desensitization was confirmed by demonstrating that CRF-stimulated cAMP accumulation was not significantly reduced following a 1-h pretreatment with 1 μM 4α-phorbol, a PMA analog that is incapable of activating PKC (Fig. 1C). Although Y79 cells were washed thoroughly at the end of the pretreatment period, basal cAMP levels were significantly increased (p < 0.001) following a 1-h exposure to PMA but not 4α-phorbol (Fig. 1).

When the time course of the desensitizing effect of PMA pretreatment was analyzed, CRF-stimulated cAMP accumulation was found to decrease progressively in Y79 cells exposed to 1 μM PMA for 5 min (22.7 ± 1.6%), 10 min (48.9 ± 2.2%), 15 min (48.8 ± 2.6%), and 30 min (58.2 ± 2.6%) (Fig. 2A). The magnitude of PMA-induced CRF1 receptor desensitization was similar in Y79 cells exposed to PMA for 30 min or 1 h (56.3 ± 1.2%) (Fig. 2A). However, the reduction in CRF-stimulated cAMP accumulation was significantly less following exposure to PMA for 3 h (42.2 ± 1.2%; p < 0.01) compared with 1 h (Fig. 2A). Although PMA pretreatment markedly reduced cAMP accumulation in Y79 cells rechallenged with CRF, it did not significantly decrease forskolin-stimulated cAMP accumulation during the same time period (Fig. 2A). When full CRF concentration-response curves were generated, the maximum CRF-stimulated cAMP accumulation was decreased ~50% in Y79 cells exposed to 1 μM PMA (14.0 pmol/106 cells) compared with control cells (28.8 pmol/106 cells) (Fig. 2B).

The time course for homologous CRF1 receptor desensitization in Y79 cells exposed to 1 μM CRF for 5 min to 3 h
revealed progressive reductions in CRF-stimulated cAMP accumulation ($p < 0.0001$) (Fig. 3). However, in contrast to the time course of PMA-induced CRF$_1$ receptor desensitization (Fig. 2A), the reduction in CRF-stimulated cAMP accumulation was significantly greater in Y79 cells exposed to 1 $\mu M$ CRF for 3 h (65.4 $\pm$ 1.8%; $p < 0.05$) compared with 1 h (49.3 $\pm$ 2.8%) (Fig. 3). The stimulation of cAMP accumulation by 50 $\mu M$ forskolin in Y79 cells desensitized by CRF did not differ from cAMP responses to forskolin in control cells (data not shown), as previously observed (Hauger et al., 1997).
Concentration-Dependent Characteristics of PMA-Induced CRF<sub>1</sub> Receptor Desensitization in Y79 Cells.

We next established the concentration-dependence of the desensitizing effect of a 15-min PMA exposure on the ability of CRF to stimulate cAMP accumulation. CRF-stimulated cAMP accumulation was first observed to decrease at 10 nM PMA (80.8 ± 2.0% control; p < 0.001) and reached a nadir at 100 nM PMA (48.1 ± 3.6% control; p < 0.001) (Fig. 4). The EC<sub>50</sub> values for the PMA-induced CRF<sub>1</sub> receptor desensitization data were 15 nM. When Y79 cells were coincubated for 15 min with 10 nM CRF (which alone did not significantly desensitize CRF<sub>1</sub> receptors) and 10 nM PMA, the magnitude of CRF<sub>1</sub> receptor desensitization (48.3 ± 2.6%; p < 0.05) was significantly greater than that caused by 15-min pretreatment with 10 nM PMA (32.8 ± 3.7%) (Fig. 5). Concentration-dependent CRF<sub>1</sub> receptor desensitization was also observed in Y79 cells exposed to PMA (0–1 μM) for 1 h (p < 0.0001; data not shown).

Effect of 1,2-Dioctanoyl-sn-glycerol (DOG) on CRF-Stimulated cAMP Accumulation. DOG structurally resembles the diacylglycerols formed when membrane phosphoinositides are hydrolyzed during G<sub>q</sub>-coupled GPCR activation, and represents a more “physiological” signal stimulating PKC translocation to the membrane. Y79 cells pretreated with 100 μM DOG for 30 min (12.7 ± 0.4 pmol/10<sup>6</sup> cells; p < 0.001) and 1 h (10.0 ± 0.3 pmol/10<sup>6</sup> cells; p < 0.001) exhibited time-dependent reductions in the magnitude of CRF-stimulated cAMP accumulation compared with control cells (17.0 ± 0.4 pmol/10<sup>6</sup> cells) (Fig. 6). The magnitude of CRF<sub>1</sub> receptor desensitization resulting from a 1-h DOG exposure (40.4 ± 2.1%; p < 0.05) was significantly greater than that observed after a 3-h DOG exposure (29.0 ± 2.7% decrease) (Fig. 6).

Expression of Protein Kinase C Isoforms in Y79 Cells. Retinoblastoma cell lysates were immunoprobed with antibodies that selectively recognize specific PKC isoforms to...
Expression of PKC exposed to 100 nM CRF (data not shown). However, no immunoreactive bands were detected with specific antibodies for PKC (Hauger et al., 1997; Dautzenberg et al., 2002). The levels of PKCα and βI proteins were depleted in Y79 cells exposed to PMA for 24 h or 48 h compared with control cells (Fig. 7, A–C). In contrast, incubating Y79 cells with 1 μM CRF for 24 h or 48 h did not decrease the expression of PKCα (data not shown) or PKCβ (Fig. 7C). This duration of CRF treatment produces >90% desensitization of retinoblastoma CRF1 receptors and a 2- to 3-fold up-regulation of GRK3 expression (Hauger et al., 1997; Dautzenberg et al., 2002).

**Effect of Down-Regulating PKC Expression on PMA-Induced CRF1 Receptor Desensitization.** Down-regulation of PKC by chronic phorbol ester treatment is another method for confirming that protein kinase C plays a role in receptor desensitization. Prolonged exposure of cells to PMA induces proteolysis, thereby depleting classical and novel, but not atypical, isoforms of PKC (Tanaka and Nishizuka, 1994). When retinoblastoma cells were pretreated with 1 μM PMA for 1 h, the magnitude of CRF-stimulated cAMP accumulation (11.4 ± 0.3 pmol/10^6 cells; p < 0.001) increased by 60% compared with the CRF-stimulated cAMP response in control cells (Fig. 8). However, a large decrement in the ability of PMA to desensitize CRF1 receptors was observed in Y79 cells chronically exposed to 1 μM PMA for 24 h (61.8 ± 4.9% of control) before 1-h PMA pretreatment and subsequent 15-min CRF stimulation (Fig. 8A). The similar magnitudes of CRF-stimulated cAMP accumulation in control Y79 cells (20.1 ± 0.6 pmol/10^6 cells) and cells subjected to the 48-h PMA exposure (22.0 ± 0.5 pmol/10^6 cells) indicated that chronic PMA treatment abolished PMA-induced CRF1 receptor desensitization (Fig. 8A). Exposure of Y79 cells to PMA for 1 h, 24 h, or 48 h did not alter forskolin-stimulated cAMP accumulation (Fig. 8, A and B). As described above, the levels of PKCα, βI, and βII proteins were depleted in Y79 cells exposed to PMA for 24 h or 48 h compared with control cells, while PKCα expression did not change (Fig. 7, A–C). Since PKC down-regulation by 48-h PMA treatment abolished PMA-induced CRF1 receptor desensitization, typical but not atypical PKC isoforms desensitize retinoblastoma CRF1 receptors without altering adenyl cyclase activity. Finally, in preliminary experiments, the magnitudes of homologous CRF1 receptor desensitization did not differ in control Y79 cells and cells in which PKC was down-regulated by 48-h PMA exposure (data not shown).
Effect of Protein Kinase C Inhibition on Homologous and Heterologous CRF1 Receptor Desensitization. The PKC inhibitors BIM (Fig. 9A) and staurosporine (500 nM; data not shown) had no effect on the homologous desensitization of retinoblastoma CRF1 receptors. A 15-min exposure to 100 nM CRF resulted in similar reductions in the magnitude of cAMP accumulation following subsequent restimulation with 100 nM CRF in Y79 cells pretreated for 30 min with vehicle (30.2 ± 2.3%) or 2 μM BIM (36.9 ± 1.8%) (Fig. 9A). In contrast, pretreating Y79 cells with BIM significantly inhibited PMA-induced CRF1 receptor desensitization (Fig. 9B). CRF-stimulated cAMP accumulation decreased in Y79 cells after a 1-h exposure to 1 μM PMA. A, data (mean ± S.E.M.) expressed as percentage of control for the stimulation of cAMP accumulation by 100 nM CRF (n = 6–16 per group) were collected in three independent experiments. By ANOVA, there were significant differences across the groups (F = 52.5, p < 0.0001). The following post hoc differences were found to be statistically significant between groups: a, p < 0.001 versus control; b, p < 0.01 versus PMA 24 h; c, p < 0.001 versus PMA 48 h; d, p < 0.001 versus PMA 24 h-forskolin. B, representative experiment for 48-h PMA exposure. Data are mean ± S.E.M. of basal and CRF- and forskolin-stimulated cAMP levels (pmol/10^6 cells) (n = 6 per group). By ANOVA, there were significant differences across the groups (F = 288.4, p < 0.0001). Significant cAMP responses to 100 nM CRF (p < 0.001) and 50 μM forskolin (p < 0.001) were observed in control cells and cells pretreated with PMA for 1, 24, and 48 h. In addition, forskolin-stimulated cAMP accumulation was significantly greater than CRF-stimulated cAMP accumulation in control and PMA-treated cells (p < 0.001). However, the 48-h PMA exposure abolished PMA-induced CRF1 receptor desensitization. The following post hoc differences were also found to be statistically significant between groups: a, p < 0.001 versus control; b, p < 0.01 versus PMA 48 h.

Effect of Protein Kinase C Inhibition on Homologous and Heterologous CRF1 Receptor Desensitization. The PKC inhibitors BIM (Fig. 9A) and staurosporine (500 nM; data not shown) had no effect on the homologous desensitization of retinoblastoma CRF1 receptors. A 15-min exposure to 100 nM CRF resulted in similar reductions in the magnitude of cAMP accumulation following subsequent restimulation with 100 nM CRF in Y79 cells pretreated for 30 min with vehicle (30.2 ± 2.3%) or 2 μM BIM (36.9 ± 1.8%) (Fig. 9A). In contrast, pretreating Y79 cells with BIM significantly inhibited PMA-induced CRF1 receptor desensitization (Fig. 9B). CRF-stimulated cAMP accumulation decreased in Y79 cells after a 1-h exposure to 1 μM PMA. A, data (mean ± S.E.M.) expressed as percentage of control for the stimulation of cAMP accumulation by 100 nM CRF (n = 6–16 per group) were collected in three independent experiments. By ANOVA, there were significant differences across the groups (F = 52.5, p < 0.0001). The following post hoc differences were found to be statistically significant between groups: a, p < 0.001 versus control; b, p < 0.01 versus PMA 24 h; c, p < 0.001 versus PMA 48 h; d, p < 0.001 versus PMA 24 h-forskolin. B, representative experiment for 48-h PMA exposure. Data are mean ± S.E.M. of basal and CRF- and forskolin-stimulated cAMP levels (pmol/10^6 cells) (n = 6 per group). By ANOVA, there were significant differences across the groups (F = 288.4, p < 0.0001). Significant cAMP responses to 100 nM CRF (p < 0.001) and 50 μM forskolin (p < 0.001) were observed in control cells and cells pretreated with PMA for 1, 24, and 48 h. In addition, forskolin-stimulated cAMP accumulation was significantly greater than CRF-stimulated cAMP accumulation in control and PMA-treated cells (p < 0.001). However, the 48-h PMA exposure abolished PMA-induced CRF1 receptor desensitization. The following post hoc differences were also found to be statistically significant between groups: a, p < 0.001 versus control; b, p < 0.01 versus PMA 48 h.

Fig. 8. Effect of down-regulating protein kinase C expression on CRF1 receptor desensitization in Y79 cells induced by a 1-h exposure to 1 μM PMA. A, data (mean ± S.E.M.) expressed as percentage of control for the stimulation of cAMP accumulation by 100 nM CRF (n = 6–16 per group) were collected in three independent experiments. By ANOVA, there were significant differences across the groups (F = 52.5, p < 0.0001). The following post hoc differences were found to be statistically significant between groups: a, p < 0.001 versus control; b, p < 0.01 versus PMA 24 h; c, p < 0.001 versus PMA 48 h; d, p < 0.001 versus PMA 24 h-forskolin. B, representative experiment for 48-h PMA exposure. Data are mean ± S.E.M. of basal and CRF- and forskolin-stimulated cAMP levels (pmol/10^6 cells) (n = 6 per group). By ANOVA, there were significant differences across the groups (F = 288.4, p < 0.0001). Significant cAMP responses to 100 nM CRF (p < 0.001) and 50 μM forskolin (p < 0.001) were observed in control cells and cells pretreated with PMA for 1, 24, and 48 h. In addition, forskolin-stimulated cAMP accumulation was significantly greater than CRF-stimulated cAMP accumulation in control and PMA-treated cells (p < 0.001). However, the 48-h PMA exposure abolished PMA-induced CRF1 receptor desensitization. The following post hoc differences were also found to be statistically significant between groups: a, p < 0.001 versus control; b, p < 0.001 versus PMA 48 h.

Fig. 9. A, effect of pretreating Y79 cells with the PKC inhibitor bisindolylmaleimide I (2 μM) for 30 min on homologous CRF1 receptor desensitization induced by 15-min exposure to 100 nM CRF. Data are mean ± S.E.M. of values expressed as percentage of control (n = 19–20 replicates per treatment group) obtained in two separate experiments. By ANOVA, there were significant differences across the groups (F = 66.7, p < 0.0001). The following post hoc differences were found to be statistically significant between groups: a, p < 0.001 versus control. B, effect of pretreating Y79 cells with the PKC inhibitor bisindolylmaleimide I (2 μM) for 30 min on PMA-induced CRF1 receptors in Y79 cells. Data are mean ± S.E.M. of values expressed as percentage of control (n = 7–17 replicates per treatment group) obtained in two separate experiments. After the 30-min pretreatment with 2 μM BIM was completed, Y79 cells were exposed to 100 nM PMA or 1 μM PMA for 15 min. By ANOVA, there were significant differences across the groups (F = 80.2, p < 0.0001). The following post hoc differences were found to be statistically significant between groups: a, p < 0.001 versus control; b, p < 0.05 versus 100 nM PMA-no PKC inhibitor; c, p < 0.001 versus 100 nM PMA-no PKC inhibitor; d, p < 0.001 versus 1 μM PMA-no PKC inhibitor.
subjected to a 15-min exposure to 100 nM (53.9 ± 1.5% of control; \( p < 0.001 \)) or 1 \( \mu \)M PMA (44.1 ± 2.6% of control; \( p < 0.001 \)) (Fig. 9B). A 30-min pretreatment with 2 \( \mu \)M BIM inhibited CRF\(_1\) receptor desensitization 56.6 ± 2.2% (\( p < 0.001 \)) and 53.5 ± 2.5% (\( p < 0.001 \)) during a 15-min exposure to 100 nM or 1 \( \mu \)M PMA, respectively (Fig. 9B).

**Effect of Protein Kinase C Inhibition on PMA-Induced CRF\(_1\) Receptor Phosphorylation.** We have previously reported that a high degree of CRF\(_1\) receptor phosphorylation develops when COS-7 cells transiently transfected with an HA-epitope-tagged CRF\(_1\) receptor cDNA are treated with CRF (Hauger et al., 2000). A 1.82 ± 0.09-fold increase (\( p < 0.001 \)) in the density of the CRF\(_1\) receptor phosphoprotein band (\( M_r \sim 70,000 \)) was again observed in transfected COS-7 cells stimulated for 5 min with 1 \( \mu \)M CRF compared with the basal phosphorylation level (data not shown). PMA-induced PKC activation in transfected COS-7 cells strongly phosphorylated the CRF\(_1\) receptor (Fig. 10A). In a total of three experiments, the magnitude of CRF\(_1\) receptor phosphorylation after a 5-min exposure to 200 nM PMA was 2.32 ± 0.21-fold greater compared with basal phosphorylation in control cells not exposed to PMA (\( p < 0.001 \)) (Fig. 10B). A 30-min pretreatment with 2 \( \mu \)M BIM completely abolished PMA-induced CRF\(_1\) receptor phosphorylation (Fig. 10). In addition, the basal level of CRF\(_1\) receptor phosphorylation in untreated control cells was significantly decreased by BIM pretreatment (\( p < 0.05 \); Fig. 10B).

**Discussion**

This study establishes that CRF\(_1\) receptors endogenously expressed in human retinoblastoma Y79 cells undergo rapid PKC-mediated desensitization. In a series of experiments, PMA (0–1 \( \mu \)M) desensitized retinoblastoma CRF\(_1\) receptors in a time- and concentration-dependent manner during a 5-min to 1-h pretreatment period. In addition, coincubation of Y79 cells for 15 min with 10 nM PMA (i.e., a concentration producing ~20% desensitization) and 10 nM CRF (which did not result in any significant desensitization) before the 15-min PMA stimulation period desensitized CRF\(_1\) receptors in an additive manner. Since forskolin-stimulated cAMP accumulation did not decrease when Y79 cells were exposed to PMA, PKC-induced CRF\(_1\) receptor desensitization does not appear to involve a direct action of protein kinase C on adenyl cyclase activity. When full CRF stimulation dose-response curves were generated, the maximum for CRF-stimulated cAMP accumulation decreased by more than 50% in Y79 cells exposed to 1 \( \mu \)M PMA for 1 h. The inability of 4\( \alpha \)-phorbol, which cannot activate PKC, to desensitize retinoblastoma CRF\(_1\) receptors during a 1-h pretreatment period confirmed the specificity of PMA-induced CRF\(_1\) receptor desensitization. Pretreatment of Y79 cells for 30 min or 1 h with DOG, a more “physiological” PKC activator, also caused a large degree of CRF\(_1\) receptor desensitization.

Protein kinase C isoforms comprise a family of serine/threonine kinases that phosphorylate many cellular proteins, including GPCRs (Tanaka and Nishizuka, 1994; Penn and Benovic, 1998; Dempsey et al., 2002; Pierce et al., 2002). Retinoblastoma cells were found to express three conventional PKCs (\( \alpha \), \( \beta \), \( \beta I \)), RACK1, which selectively binds PKC\( \beta I I \) (Dempsey et al., 2002), and one atypical PKC (\( \lambda \)). Diacylglycerols and phorbol esters bind to the N-terminal C1 regulatory domain of PKCa, \( \beta I \), and \( \beta I I \) (but not PKCa) and down-regulate these conventional PKC proteins when cells are chronically exposed to PKC activators (Tanaka and Nishizuka, 1994). When the duration of PMA or DOG exposure was extended to 3 h in our study, the magnitude of CRF\(_1\) receptor desensitization was significantly less than that occurring with a 1-h pretreatment, presumably due to in-

**Fig. 10.** Effect of the protein kinase C inhibitor bisindolylmaleimide I on PMA-stimulated HA-CRF\(_1\)-receptor phosphorylation. A, after a 30-min pretreatment period with vehicle or 2 \( \mu \)M BIM was completed in a representative experiment, \(^{32}\)P-labeled HA-CRF\(_1\)-R-expressing COS-7 cells were exposed to media (control) or 1 \( \mu \)M PMA for 5 min. Phospho-HA-CRF\(_1\), receptors were immunoprecipitated and resolved by SDS-PAGE. The 60,000 to 70,000 \( M_r \) band representing the phosphorylated CRF\(_1\) receptor was 2.5-fold greater in PMA-treated cells (lane 3) compared with the band obtained from cells exposed to vehicle (lane 1). BIM pretreatment abolished PMA-induced HA-CRF\(_1\) receptor phosphorylation (lane 4). B, data (mean ± S.E.M.) from three separate experiments are presented as percentage of increase above basal HA-CRF\(_1\) receptor phosphorylation in control cells (transfected with HA-CRF\(_1\) receptor cDNA but not exposed to PMA). Pretreatment of transfected cells with 2 \( \mu \)M BIM for 30 min lowered basal receptor phosphorylation and blocked the ability of PMA to stimulate receptor phosphorylation. By ANOVA, there were significant differences across the groups (\( F = 35.5, p < 0.0001 \)). The following post hoc differences were found to be statistically significant between groups: a, \( p < 0.001 \) versus PMA-no PKC inhibitor; b, \( p < 0.05 \) versus control-no PKC inhibitor; c, \( p < 0.001 \) versus PMA-BIM.
increased proteolytic degradation of PKC proteins. Subsequent experiments confirmed this possibility by demonstrating that PMA-induced CRF<sub>1</sub> receptor desensitization was abolished in Y79 cells where PKC<sub>α</sub> and PKC<sub>β</sub>I protein levels were depleted by a 48-h PMA exposure. PKC<sub>α</sub> protein levels were not reduced in Y79 cells chronically exposed to PMA. Chronic PMA treatment can also down-regulate expression of the novel isoform PKC<sub>δ</sub> (Tanaka and Nishizuka, 1994), which is expressed in Y79 cells. Therefore, our data indicate that α-, β-, and possibly δ-isoforms of PKC desensitize retinoblastoma CRF<sub>1</sub> receptors.

BIM acts as a competitive inhibitor at the ATP binding site within the catalytic domains of many PKC isoforms including PKC<sub>α</sub>, PKC<sub>β</sub>, PKC<sub>δ</sub>, PKC<sub>ε</sub>, and PKC<sub>ζ</sub> (Tanaka and Nishizuka, 1994). Although 2 μM BIM pretreatment did not alter homologous CRF<sub>1</sub> receptor desensitization, it markedly inhibited PMA-induced CRF<sub>1</sub> receptor desensitization. This suggests that a PKC-dependent mechanism contributes to heterologous, but not homologous, desensitization of CRF<sub>1</sub> receptors.

CRF<sub>1</sub> receptors in brain and transfected mouse fibroblast cells can couple to both G<sub>α</sub> and G<sub>q</sub> proteins, thereby activating both cAMP and phospholipase C pathways (Dieterich et al., 1996; Hillhouse et al., 2002). In Leydig cells and placenta, CRF<sub>1</sub> receptors may signal via G<sub>q</sub>-mediated stimulation of phospholipase C and formation of IPs without generating an intracellular cAMP-dependent signal (Ulisse et al., 1990; Karteris et al., 2000). Taken together, these findings suggest that the CRF<sub>1</sub> receptor can couple to G<sub>q</sub> and signal via the protein kinase cascade in certain cell types (Kiang et al., 1994; Dautzenberg and Hauger, 2002). However, inositol phosphate generation was not increased in Y79 cells stimulated with CRF and inhibition of PKC in Y79 cells by BIM pretreatment failed to block homologous CRF<sub>1</sub> receptor desensitization. Thus, it seems likely that the CRF<sub>1</sub> receptor expressed in retinoblastoma cells couples to G<sub>q</sub>, but not to G<sub>q'</sub>. Similar findings have been observed for the pituitary adenylate cyclase-activating polypeptide type 1 receptor (PAC<sub>1</sub>). The PAC<sub>1</sub> receptor exclusively couples to G<sub>q</sub> in Y79 cells (Olianas et al., 1996; Dautzenberg et al., 1999), while the PAC<sub>1</sub> receptor couples to G<sub>q</sub> in other cells (Harmar et al., 1998).

We determined whether an HA-epitope-tagged CRF<sub>1</sub> receptor recombinantly expressed in COS-7 cells could be phosphorylated by protein kinase C to establish a possible mechanism for PMA-induced CRF<sub>1</sub> receptor desensitization. A high degree of CRF<sub>1</sub> receptor phosphorylation was detected in transfected COS-7 cells during a 5-min exposure to PMA—an effect that was completely abolished by 30-min pretreatment with BIM. We previously observed that CRF exposure, but not forskolin-induced PKA activation or ionomycin-induced stimulation of Ca<sup>2+</sup>/calmodulin-dependent kinases, causes phosphorylation of the CRF<sub>1</sub> receptor in COS-7 cells, presumably due to a G protein receptor kinase mechanism (Hauger et al., 2000). Similarly, stimulation of PKA activity by forskolin or dibutyryl cAMP failed to desensitize CRF<sub>1</sub> receptors in Y79 and IMR-32 cells (Dautzenberg et al., 2001a; Roseboom and Kalin, 2001).

Recently, mice with a targeted deletion of the PKC<sub>β</sub> gene were found to exhibit a deficit in fear conditioning (Weever et al., 2000). In BALB/c mice, context-dependent fear conditioning was augmented by injecting CRF or activating PKC in the hippocampus, while pretreatment with a PKC inhibitor impaired acute stress-induced enhancement of context-dependent fear conditioning (Blank et al., 2002, 2003). PKC inhibitors also block CRF-induced increases in firing rates of hippocampal neurons in BALB/c mice (Blank et al., 2003). Consequently, PKC-mediated CRF<sub>1</sub> receptor signaling may contribute to the expression of contextual fear. We have previously hypothesized that deficient GRK-mediated CRF<sub>1</sub> receptor desensitization may trigger long-term psychopathology by increasing the sensitivity of and/or prolonging agonist-stimulated CRF<sub>1</sub> receptor signaling in brain pathways mediating fear and anxiety (Dautzenberg and Hauger, 2002). The present data suggest that abnormalities in PKC-mediated phosphorylation and desensitization of CRF<sub>1</sub> receptors may also result in CRF<sub>1</sub> receptor supersensitivity and sensitization of brain CRF neurotransmission, thereby reducing the activation threshold and increasing the magnitude and duration of fear and anxiety responses.

In conclusion, we have demonstrated that rapid desensitization of retinoblastoma CRF<sub>1</sub> receptors occurred when protein kinase C is activated. The presence of two potential PKC phosphorylation sites (Ser<sup>386</sup> and Ser<sup>408</sup>) in the C-terminal cytoplasmic tail of the CRF<sub>1</sub> receptor suggests that the phosphorylation of the CRF<sub>1</sub> receptor by protein kinase C observed in this study may mediate this desensitization process (Chen et al., 1993; Dautzenberg et al., 2001b). However, depending on the cellular background, PKC-mediated CRF<sub>1</sub> receptor desensitization may occur via heterologous cross-talk with one or more G<sub>q</sub>-coupled GPCRs or via a homologous mechanism whereby the agonist-activated CRF<sub>1</sub> receptor couples to the G<sub>q</sub> protein and subsequently activates its phosphorylation by PKC. In a recent study it was found that activation of oxytocin receptors causes heterologous desensitization of CRF-stimulated cAMP accumulation in human myometrial cells via a PKC-dependent mechanism (Grammatopoulos and Hillhouse, 1999). Based on previous reports of cross-talk between receptor signaling pathways (Selbie and Hill, 1998; Budd et al., 1999; Thakker and Standifer, 2002), activation of heterologous GPCRs may result in cross-phosphorylation, desensitization, and possibly internalization of CRF<sub>1</sub> receptors, thereby shifting the stress response to other receptor mechanisms. Future studies will be directed at the identification of GPCRs that desensitize retinoblastoma CRF<sub>1</sub> receptors via PKC-mediated cross-talk.

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