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Antisense Oligonucleotides Targeting Human Protein Kinase C-α Inhibit Phorbol Ester-Induced Reduction of Bradykinin-Evoked Calcium Mobilization in A549 Cells

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SUMMARY

Regulation of the bradykinin-evoked increase in intracellular Ca^{2+} concentration by protein kinase C (PKC)-α was investigated in A549 human lung carcinoma cells. Bradykinin, a potent and selective kinin B_2 receptor agonist, induces calcium mobilization in a concentration-dependent fashion in this cell line. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), a potent activator of PKC, is known to reduce the amplitude of agonist-induced calcium mobilization in various cell lines. Because PKC-α is a major PKC isozyme in A549 cells, we investigated whether this isozyme plays a role in this process. A 20-mer phosphorothioate oligonucleotide targeting the 3′-untranslated region of the human PKC-α mRNA, which contains 2′-methoxyethyl modifications incorporated into the 5′ and 3′ segments of the oligonucleotide, was used to assess the putative role of PKC-α in the receptor regulation. ISIS 9606 reduced PKC-α mRNA for $\geq$72 hr after the initial treatment and the reduction was concentration dependent, whereas the mismatch control, ISIS 13009, had no effect. Concentrations of ISIS 9606 of 150 nM specifically reduced the level of immunoreactive PKC-α protein by 66.3 $\pm$ 2.5% at 72 hr after treatment, without an effect on immunoreactive PKC-δ protein. This reduction in PKC-α was sufficient to inhibit the reduction of bradykinin-induced calcium mobilization by TPA. This finding is corroborated by the use of staurosporine, a nonselective PKC inhibitor, that prevented the effect of TPA. These results suggest that PKC-α is involved in kinin B_2 receptor regulation by phorbol esters in A549 cells.

It is well established that activation of PKC regulates receptor-mediated events linked to phosphoinositide hydrolysis, generating inositol-1,4,5-trisphosphate, which induces subsequent increases in $[Ca^{2+}]_i$ (1–3). In many instances, cells treated for a short period of time ($\leq$30 min) with PKC activators such as TPA display dramatically reduced agonist-induced calcium mobilization, while a longer incubation (>6 hr) can enhance the response (2–4). Activation of PKC by diacylglycerol or phorbol esters is thought to involve translocation of the protein from the cytosol to the membrane, regulating receptor functions (5). However, little is known about the roles of individual PKC isozymes in this process.

Many inhibitors of PKC have been used to understand and better characterize the role of PKC in cell physiology. Inhibitors such as aminoacridines (6), sphingolipids (7), staurosporine and analogs (8, 9), calphostin C (10), bisindolylmaleimides (11), and isoquinolinesulfonamides (12) exhibit some specificity for PKC. For example, previous reports demonstrated that staurosporine reversed receptor desensitization induced by phorbol esters in various cell types (2, 13). However, PKC is a family of at least 12 isozymes, all with closely related structures but differing individual properties and tissue expression (5, 14). These isozymes have been subdivided on the basis of structural similarities into conventional (PKC-α, -βI, -βII, and -γ), new (PKC-δ, -ϵ, -η, and -θ), atypical (PKC-ζ, -λ, and -ι), and μ-like (PKC-μ) PKC isoforms. Considerable evidence exists suggesting that individual isozymes possess different functions in different cell types. The roles played by individual PKC isozymes in a number of cellular functions, including cell differentiation and proliferation (15–17), transmitter release and exocytosis (18), regulation of phospholipase D, C, and A_2 activity (4, 19–21), and mitogenesis (21), have been studied. Methods used to characterize the role of distinct PKC isozymes in these biochemical assays include overexpression of PKC isozyme cDNAs, Northern and Western blot techniques with PKC isozyme-specific probes for tracking the localization and redistribution of the PKC isozymes after cell stimuli, and intracellular delivery of anti-PKC antibodies into transiently permeabilized cells. Nevertheless, progress in determining the roles of each of the isozymes in various biological processes has been hindered by the lack of isozyme-specific PKC inhibitors.

ABBREVIATIONS: PKC, protein kinase C; G3PDH, glycerol-3-phosphate dehydrogenase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $[Ca^{2+}]_i$, intracellular calcium concentration; AM, acetoxymethyl ester.

To overcome this lack of selectivity, antisense oligonucleotides have been identified that selectively inhibit PKC-α mRNA and protein expression (22–25). These first-generation antisense oligonucleotides were 20-mer phosphorothioate oligodeoxynucleotides targeted to different regions of the PKC-α cDNA that have been shown to specifically inhibit the mRNA and protein expressions of PKC-α without nonspecific inhibition of PKC-δ, PKC-ϵ, PKC-η, and PKC-ζ isozymes (22–25). Improvements in oligonucleotide stability and potency have subsequently been achieved through the development of 2′-*O*-propyl chimeric oligonucleotides targeted against the murine PKC-α cDNA (24). We adopted a similar strategy through the creation of chimeric oligonucleotides containing 2′-methoxyethyl modifications in the wings (3′ and 5′ portions) and an oligodeoxynucleotide gap in the center designed to serve as a substrate for RNase H. Because these second-generation antisense oligonucleotides contain phosphorothioate linkages throughout the molecule, they are extremely stable and more potent than phosphorothioate oligodeoxynucleotides (26).

On the basis of translocation patterns of individual PKC isozymes after TPA exposure and correlation of these findings to effects on phospholipase C activity, a previous study concluded that PKC-α and PKC-δ mediate phorbol ester-induced reduction in bradykinin-evoked inositol-1,4,5-triphosphate generation in astrocytes (4). To provide additional insight into this process, a direct approach, with antisense oligonucleotides, was used to evaluate the putative roles of PKC-α in mediating the phorbol ester-induced reduction of calcium mobilization induced by bradykinin in A549 human lung carcinoma cells. We have found that selective depletion of PKC-α protein in A549 cells partially restores calcium mobilization induced by bradykinin when cells are pretreated with phorbol esters. This suggests that PKC-α plays a role in the regulation of bradykinin B_2 receptors in this cell type and that appropriately designed antisense oligonucleotides may be useful tools in the dissection of the roles of the multiple isozymes.

Materials and Methods

Cell culture. Human A549 lung carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium containing 1 g of glucose/liter (GIBCO, Grand Island, NY). The media was supplemented with 10% fetal bovine serum and antibiotics (0.1 mg/ml penicillin and 0.1 mg/ml streptomycin; GIBCO). Cells were routinely passaged at 85–95% confluency in T-175 flasks. The cells were plated onto 100-mm^2 culture dishes or T-75 flasks for Western or Northern blot analysis, respectively. Cells were maintained in T-175 flasks for calcium mobilization and PKC assays.

Oligonucleotide synthesis. 2′-*O*-Methoxyethyl-substituted oligonucleotides and phosphorothioate oligodeoxynucleotides were prepared via conventional phosphoroamidite methodology using controlled pore glass as the solid support with an Applied Biosystems (Foster City, CA) 380B automated synthesizer. A 3′-succinylated 2′-*O*-methoxyethyl oligonucleotide was attached to the controlled pore glass after activation with benzotriazol-1-yl-oxytripyrrolidinephosphonium hexafluorophosphate. After detritylation, phosphoroamidites were coupled using 1-ethylthiotetrazole as the catalyst. Typical stepwise coupling efficiencies were >99%. Phosphorothioate linkages were produced using Beaucage reagent for sulfurization. Phosphorothioate linkages were incorporated through oxidation with a 10% solution of *t*-butylhydroperoxide in acetonitrile. Oligonucleotides were deprotected in concentrated ammonium hydroxide at 55° for 24 hr and purified by precipitation from ethanol or reversed-phase high performance liquid chromatography on a C-18 column using an acetonitrile gradient. Full-length product comprised >85% of the sample, as determined using capillary gel electrophoresis. Molecular masses were measured via electrospray mass spectrometry, and observed values were within 0.02% of the calculated values. Mass spectrometry also demonstrated that the mole fraction of molecules containing all phosphorothioate linkages to those molecules containing a single phosphodiester linkage was >0.92. Oligonucleotide sequences and their hybridization thermodynamic properties are listed in Table 1. The melting temperature of each oligonucleotide was determined in triplicate as previously described (27).

Treatment of cells with oligonucleotides. A549 cells at 85–90% confluency were washed once with prewarmed Dulbecco's modified Eagle's medium. A solution containing 15 μg/ml *N*-[1-(2,3-dioleyloxy)propyl]-*n*,*n*,*n*-triethylammonium chloride/dioleoylphosphatidylethanolamine (GIBCO) and oligonucleotides, when indicated, was then added to the flasks. The cells were incubated at 37° for 4 hr, and the *N*-[1-(2,3-dioleyloxy)propyl]-*n*,*n*,*n*-triethylammonium chloride/dioleoylphosphatidylethanolamine/oligonucleotide mixture was aspirated off of the cells and replaced with media containing 0.4% fetal bovine serum.

Measurement of PKC mRNA levels. PKC-α mRNA expression in A549 cells was evaluated as previously described (27). Briefly, the total cellular RNA of cells treated with oligonucleotides was isolated by lysis in 4 M guanidinium isothiocyanate followed by a cesium chloride gradient. Total RNA (20–25 ng) was resolved on 1.2% agarose gels containing 1.1% formaldehyde and transferred to nylon membranes (Hybond). The blots were prehybridized in Quikhyb solution (Stratagene, La Jolla, CA) for 1 hr at 68° and probed using a bovine PKC-α cDNA probe (American Type Culture Collection) that was ^{32}P-radiolabeled with [α-^{32}P]dCTP by random priming (Promega, Madison, WI) according to the manufacturer's protocol. The membranes were routinely stripped (through boiling in 0.1% standard saline citrate/0.1% sodium dodecyl sulfate for 2 min) and then reprobed with a radiolabeled human G3PDH probe to confirm equal loading. Hybridizing bands were visualized and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

TABLE 1

Sequences and melting temperatures of oligonucleotides designed to hybridize to human PKC-α mRNA

Oligonucleotide	Sequence and modification	Number of mismatches	Melting temperature (°)
3521	GTT CTC GCT GGT GAG TTT CA	0	52.1
13032	GTT CTC GCC GGT GAG TTT CA	1	46.1
13031	GTT CTC GCT CGT GAG TTT CA	1	39.7
13030	GTT CTA GCT GGT GCG TTT CA	2	25.5
13029	GTT CTA GCT CGT GCG TTT CA	3	N.C.
13028	GTT CGA GCT GGT GCT TTT CA	4	~22
9606	**GTT CTC** GCT GGT GA**G TTT CA**	0	64.8
13009	**GGT TTT** ACC ATC GGT **TCT GG**	13	N.D.

Underlined bases are the mismatched bases compared with 3521. Bold bases are the 2′-*O*-methoxyethyl nucleotides. N.C., noncooperative. N.D., not determined.

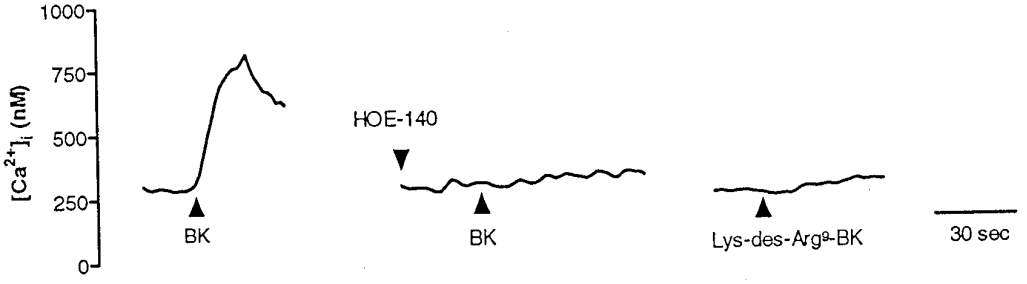

Fig. 1. Pharmacological characterization of the kinin-evoked increase in $[Ca^{2+}]_i$ in human lung carcinoma A549 cells. Cells were stimulated with 1 μM of bradykinin or Lys-des-Arg^9-bradykinin. The kinin B_2 antagonist HOE-140 (70 nM) was added 1 min before challenge with agonist.

Immunoblotting of PKC isozymes. After oligonucleotide treatment, cells were washed once with ice-cold phosphate-buffered saline. Proteins were extracted in 250 μl of lysis buffer [20 mM Tris, pH 7.4, 1% (v/v) Triton X-100, 5 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 50 mM NaF, 10 mM Na_2HPO_4] supplemented with leupeptin (2 μg/ml) and aprotinin (1 μg/ml) at 4°. The protein content of the samples was determined with the Bradford protein assay (BioRad, Hercules, CA) using bovine serum albumin as standard. Samples were electrophoresed through a 12% acrylamide gel and then electroblotted. The expressions of PKC-α (79 kDa) and G3PDH (33 kDa) were simultaneously determined by use of anti-PKC-α (1:2,000; Upstate Biotechnology, Lake Placid, NY) and anti-G3PDH (1:50,000; Advanced ImmunoChemical, Long Beach, CA) monoclonal antibodies.

A polyclonal antibody was used to determine PKC-δ (1:750; Santa Cruz Biotechnology, Santa Cruz, CA). After a minimum of a 2-hr incubation with the primary antibody, the membranes were incubated with either 5 μCi of ^{125}I-labeled goat anti-mouse or ^{125}I-labeled goat anti-rabbit antibodies (ICN Radiochemicals, Costa Mesa, CA) for 1 hr. Hybridizing bands were visualized and quantified using a PhosphorImager.

Calcium mobilization assay. The assay was performed as previously described with several modifications (28). Cells were harvested using a phosphate-buffered saline/EDTA (1 mM) solution, and washed twice in the calcium buffer (10 mM HEPES, pH 7.4; 140 mM NaCl, 10 mM glucose, 5 mM KCl, 1 mM $MgSO_4$, 1.8 mM $CaCl_2$), and resuspended at 1.5×10^6 cells/ml. Fura-2/AM (2 μM) and Pluronic F-127 (0.02%; Molecular Probes, Eugene, OR) were added to the suspended cells for a minimum of 20 min at room temperature. The latter reagent is added to ensure effective loading. Just before the assay, 1 ml of cell suspension was centrifuged for 10 sec, resuspended in 2 ml of media without Fura-2/AM and Pluronic F-127, and transferred into a quartz cuvette. Experiments were performed using a Perkin-Elmer Cetus LS-50 fluorimeter (Norwalk, CT). For each determination, cells were maintained at 25° and with constant stirring. Each sample was exposed to a single concentration of agonist. Thus, to avoid receptor desensitization, repetitive estimations were not made using the same cell suspension. Cells were subjected to excitation at two wavelengths, 340 and 380 nm, and the emitted light was collected at the photomultiplier at 505 nm. The 340/380 nm ratio of the fluorescence due to excitation was calculated by the analyzer. The determination of R_{max} and R_{min} values (see below) for the calibration of $[Ca^{2+}]_i$ was performed by inclusion of 20 μM ionomycin and then 6.25 mM EGTA, pH 8.5. Measurements were corrected for autofluorescence by adding 20 μM ionomycin and 5 mM $MnCl_2$ to a aliquot of cells. The $[Ca^{2+}]_i$ levels at rest as well as at the maximal increase evoked after the addition of bradykinin were then calculated according to the formula $[Ca^{2+}]_i = K_D \times (R - R_{min})/(R_{max} - R) \times Sf_2/Sb_2$ (29). K_D (224 nM) is the affinity for Fura-2 binding to Ca^{2+}. The R values denote the ratio of fluorescence or relative fluorescence: R is the measured cellular ratio, R_{max} is the ratio determined in Ca^{2+}-saturating intracellular Fura-2 (in the presence of ionomycin), and R_{min} is the ratio determined by chelating all intracellular calcium by EGTA. The Sf_2 and Sb_2 values are 380 nm excitation signal in the absence and presence of saturating concentrations of Ca^{2+}, respectively, which is determined with the fluorimeter.

The concentration-response (peak $[Ca^{2+}]_i$) curves to bradykinin were evaluated by successive incubations, each containing similar number of cells. Concentration-response curves were quantified by taking aliquots from the same stock of cells loaded with Fura-2. The specificity of bradykinin for the kinin B_2 receptor was assessed by adding 70 nM HOE-140 at 1 min before the addition of bradykinin. The kinetics of TPA regulation of the bradykinin calcium mobilization were assessed. TPA (500 nM) was initially added to a stock of

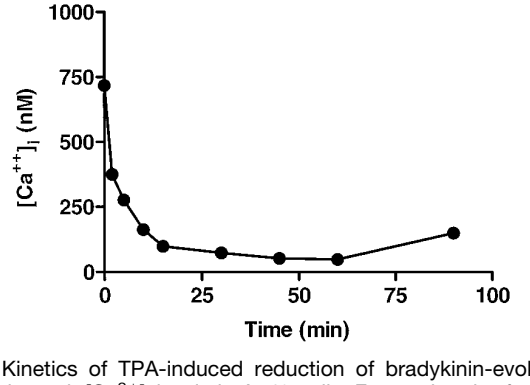

Fig. 2. Kinetics of TPA-induced reduction of bradykinin-evoked increases in peak $[Ca^{2+}]_i$ levels in A549 cells. From a batch of cells, an aliquot of cell suspension was challenged with bradykinin (100 nM) to initially determine (time zero) the peak $[Ca^{2+}]_i$ level, which was attained 15–20 sec after the challenge with the agonist. TPA (500 nM) was then added to the batch of cells, and an aliquot of the cell suspension was taken at indicated times and challenged with bradykinin to measure the peak $[Ca^{2+}]_i$ levels. Values are single determinations and are representative of two experiments.

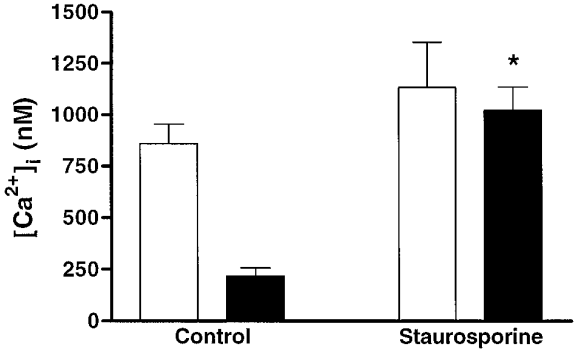

Fig. 3. Effect of staurosporine on TPA-induced reduction of bradykinin-evoked increases in peak $[Ca^{2+}]_i$ levels in A549 cells. Staurosporine (300 nM) was added to cells during Fura-2 loading. Cells are then treated with bradykinin alone (100 nM; *open bars*) or pretreated with TPA (500 nM; *solid bars*) 15 min before the addition of bradykinin. Values are mean ± standard error of seven determinations. The effect of staurosporine was compared with control by Student's *t* test: *, $p < 0.001$.

cells loaded with Fura-2. Aliquots were then taken at the indicated time points and then challenged with 1 μM of bradykinin. In subsequent experiments, TPA (500 nM) was added to cells 15 min before the addition of bradykinin (100 nM), at the time at which the effect of TPA was determined to be maximum. In some experiments, staurosporine (300 nM) was added during Fura-2 loading to assess the effect of a nonspecific PKC inhibitor on TPA-induced reduction of bradykinin-evoked calcium mobilization. Likewise, the effect of the oligonucleotides on this process was evaluated by harvesting cells 72 hr after oligonucleotide treatment (150 nM).

Reagents. Bradykinin, Lys-des-Arg^9-bradykinin, phenylephrine, acetylcholine, 5-hydroxytryptamine, endothelin-1, histamine, Fura-2/AM, angiotensin II, vasopressin, TPA, ionomycin, and EGTA were purchased from Sigma Chemical (St. Louis, MO). HOE-140 was purchased from Peninsula Laboratories (Belmont, CA).

Statistical analysis. Experimental values are reported as mean ± standard error. Comparison of mean values was performed by one-way analysis of variance followed by Dunnett's test or by Student's *t* test.

Results

Pharmacological characterization of calcium mobilization in A549 cells. To study the effects of selective reduction of PKC-α protein on receptor-mediated regulation of calcium levels, we screened various agonists for their ability to induce calcium mobilization in human lung carcinoma A549 cells. The following agonists were found to be inactive in inducing calcium mobilization in A549 cells: phenylephrine, acetylcholine, 5-hydroxytryptamine, endothelin-1, histamine, angiotensin II, and vasopressin (results not shown). Of the agents tested, only bradykinin, a potent and selective kinin B_2 receptor agonist, elicited an increase in $[Ca^{2+}]_i$. The peak increase was routinely attained 15–20 seconds after challenge with the agonist (Fig. 1). Kinin receptors are classified into the B_1 and B_2 types (30). The lack of effect of Lys-des-Arg^9-bradykinin, a kinin B_1 receptor agonist, and the antagonism of HOE-140, a very potent and selective antagonist of the kinin B_2 receptors (31), on bradykinin-induced calcium mobilization confirmed that A549 cells express solely kinin B_2 receptors (Fig. 1).

Effects of TPA and staurosporine on bradykinin-induced calcium mobilization. Bradykinin elicited a concentration-dependent increase in $[Ca^{2+}]_i$ A549 cells (results not shown), which was consistent with previous reports (32). Concentrations of 100 nM bradykinin consistently resulted in maximal elevations in $[Ca^{2+}]_i$, and this effect was partially prevented when cells were pretreated with TPA (Fig. 2). TPA (500 nM) treatment reduced calcium mobilization induced by bradykinin in a time-dependent manner. Maximal reduction occurred after 15 min and was maintained for ≥90 min. These experimental conditions were used to determine the effect of PKC inhibitors on the regulation of the kinin receptor.

Staurosporine (300 nM), a nonselective PKC inhibitor, was added to cells before the introduction of bradykinin and/or TPA (Fig. 3). The inhibitor had no effect on the maximal calcium mobilization induced by bradykinin. However, staurosporine almost completely reversed the effect of TPA. These data suggest that PKC is involved in kinin B_2 receptor regulation in A549 cells.

Reduction of PKC-α mRNA and protein levels by antisense oligonucleotides. To assess the possible roles of the PKC-α isozyme in the regulation of calcium mobilization by phorbol esters, we specifically reduced PKC-α mRNA and protein expression using antisense oligonucleotides. To provide proof of mechanism, the effects of ISIS 3521 (22) were compared with those of a series of length-matched oligonucleotides that contain a increasing number of mismatches (Table 1 and Fig. 4). The gradual increase in the number of base mismatches decreased the melting temperature of the oligonucleotides for the complementary sense strand (Table 1) and the potency of PKC-α mRNA reduction (Fig. 4). Oligonucleotides containing two or more mismatches had a minimal effect on the PKC-α mRNA expression. ISIS 9606 is 20-mer chimeric phosphorothioate oligonucleotide targeting the same site as ISIS 3521 at the 3′-untranslated region coding for human PKC-α. The inclusion of the 2′-*O*-methoxyethoxy modifications resulted in an increase in oligonucleotide melting temperature to 64.8°. Treatment of A549

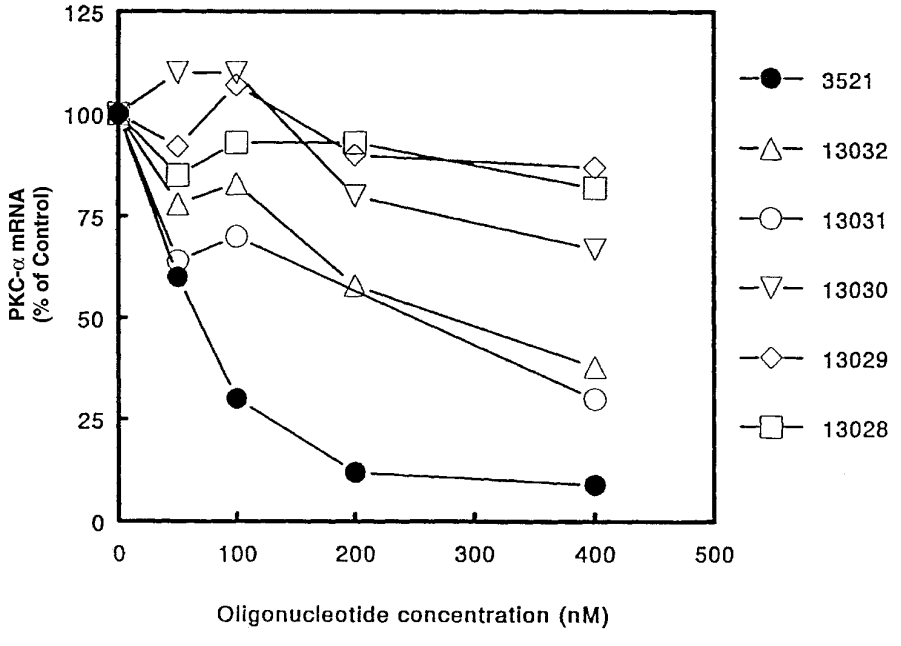

Fig. 4. Effect of base-mismatched oligonucleotides on the concentration-effect relationship for the reduction in PKC-α mRNA expression 24 hr after oligonucleotide treatment. Levels of PKC-α from gels that were quantified with a PhosphoImager and expressed as percentage of control after normalization with G3PDH. Values are the average of two determinations.

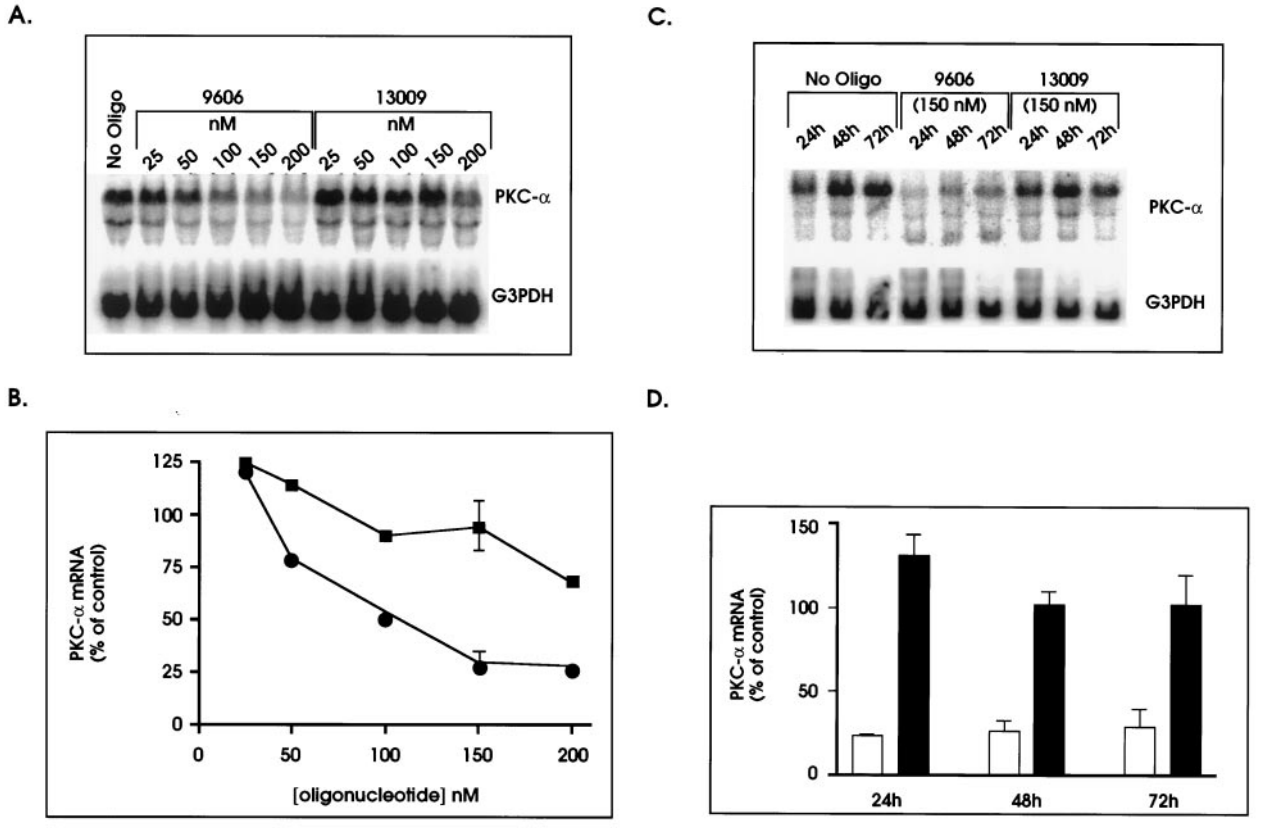

Fig. 5. Inhibition of PKC-α mRNA expression in A549 cells. A and B, Concentration-effect relationship for the reduction in PKC-α mRNA expression 24 hr after oligonucleotide treatment. A, Representative blots of cells treated with ISIS 9606 or ISIS 13009. *Top bands*, PKC-α mRNA transcripts. *Bottom band*, G3PDH mRNA transcripts, demonstrating equal loading in each lane. B, Levels of PKC-α from gels that were quantified with a PhosphoImager, normalized for G3PDH loading, and expressed as percentage of control. Values are the average of two determinations, except for concentration of 150 nM, which is the mean ± standard error of five determinations. ●, ISIS 9606. ■, ISIS 13009. C and D, Kinetic analysis of the reduction in PKC-α mRNA expression by oligonucleotides. C, Representative blots of cells treated with 150 nM of ISIS 9606 or ISIS 13009 for 24, 48, and 72 hr. *Top bands*, PKC-α mRNA transcripts. *Bottom band*, G3PDH mRNA transcripts, demonstrating equal loading in each lane. D, Levels of PKC-α from the above gels that were quantified with a PhosphorImager, normalized for G3PDH loading, and expressed as percentage of control. Values are mean ± standard error of three determinations. *Open bars*, ISIS 9606; *solid bars*, ISIS 13009.

cells with ISIS 9606 reduced the expression of both the 4-kb and the 8.5-kb species of PKC-α mRNA in a concentration-dependent fashion after 24 hr (Fig. 5, A and B). At concentrations of <200 nM, ISIS 13009, a 13 base mismatch control of ISIS 9606, did not alter the levels of PKC-α transcripts. For subsequent experiments, oligonucleotide concentrations were maintained at 150 nM to avoid nonspecific effects, and several determinations were performed at 150 nM to confirm the specificity of the oligonucleotides in our system. The reduction in PKC-α transcripts by ISIS 9606 was persistent up to 72 hr with no effect of ISIS 13009 (Fig. 5, C and D). None of the oligonucleotides tested affected G3PDH mRNA levels, demonstrating selectivity for the targeted mRNA (Fig. 5).

PKC-α protein (79 kDa) levels were not reduced until 48 hr after the addition of ISIS 9606 at a concentration of 150 nM. The maximal reduction in protein expression occurred after 72 hr (Fig. 6, A and B), whereas no further decrease in protein concentration was observed after 96 hr (results not shown). The mismatch control, ISIS 13009, did not alter protein levels at the concentrations tested. The effects of ISIS 9606 at 72 hr were concentration dependent, with a IC_{50} value of 100–130 nM (Fig. 6, C and D). The maximal PKC-α protein reduction obtained was 66.3 ± 2.5 (eight determinations) at 150 nM, and further increase in the oligonucleotide concentration did not increase the reduction in protein. G3PDH protein (33 kDa) levels were not affected by the oligonucleotides, demonstrating selectivity for the targeted protein (Fig. 6). ISIS 9606 (150 nM) was shown to be selective for the immunoreactive PKC-α and did not affect the expression of PKC-δ, whereas ISIS 13009 was inactive (Fig. 6E).

Effect of PKC-α reduction on TPA regulation of calcium mobilization. Cells were exposed to oligonucleotides as described in Materials and Methods, and the calcium mobilization assay was performed at 72 hr after treatment, when PKC-α protein reduction by ISIS 9606 was maximal. The oligonucleotides (150 nM) did not alter the maximal calcium mobilization induced by bradykinin (100 nM) (Fig. 7). In all cases, TPA significantly reduced bradykinin-induced peak $[Ca^{2+}]_i$ levels, but the reduction was less with 9606. However, ISIS 9606 (150 nM) treatment prevented a significant fraction of the inhibition induced by TPA (Fig. 7). The selectivity of the oligonucleotide was demonstrated by the lack of effect of ISIS 13009 (150 nM). These results indicate that reduction of PKC-α protein expression inhibits the effects of phorbol esters on bradykinin B_2 receptor-mediated calcium mobilization.

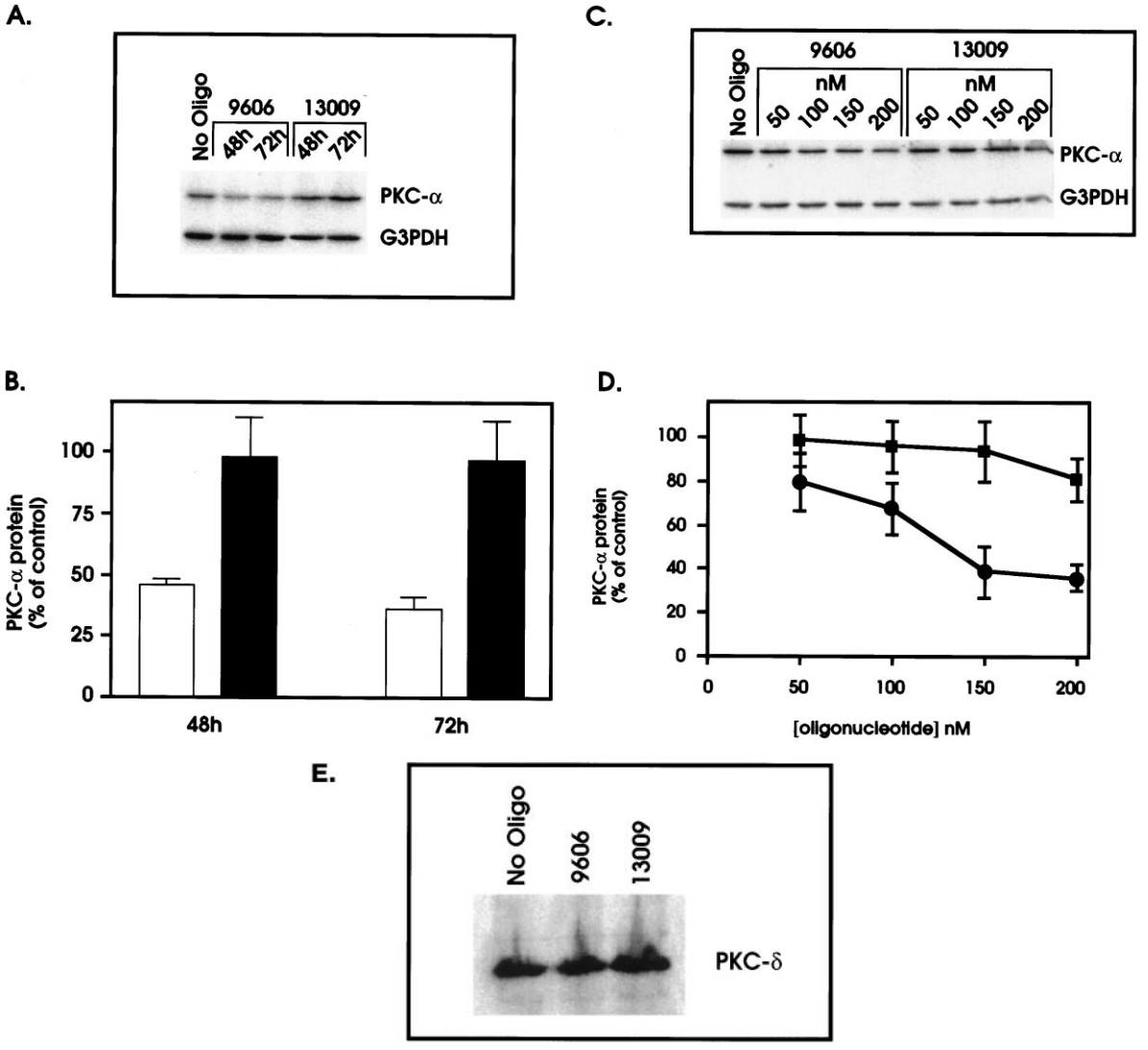

Fig. 6. Inhibition of immunoreactive PKC-α protein expression in A549 cells. A and B, Kinetic analysis of the reduction in PKC-α protein expression by oligonucleotides. A, Representative blots of cells treated with 150 nM ISIS 9606 or ISIS 13009 for 48 and 72 hr. *Top band*, immunoreactive PKC-α isozyme. *Bottom band*, immunoreactive G3PDH, demonstrating equal loading in each lane. B, Levels of PKC-α from the above gels that were quantified with a PhosphorImager, normalized for G3PDH loading, and expressed as percentage of control. Values are mean ± standard error of four determinations. *Open bars*, ISIS 9606; *solid bars*, ISIS 13009. C and D, Concentration-effect relationship for the reduction in PKC-α protein expression by oligonucleotides. C, Representative blots of cells treated with ISIS 9606 or ISIS 13009 for 72 hr. *Top band*, immunoreactive PKC-α isozyme. *Bottom band*, immunoreactive G3PDH, demonstrating equal loading in each lane. D, Levels of PKC-α from the above gels that were quantified with a PhosphorImager, normalized for G3PDH loading, and expressed as percentage of control. Values are mean ± standard error of three to six determinations. ●, ISIS 9606. ■, ISIS 13009. E, Effect of ISIS 9606 on the expression of PKC-δ in A549 cells. A549 cells were treated with 150 nM oligonucleotides, and proteins were extracted after 72 hr.

Discussion

The human lung carcinoma cell line (A549) is reported to exhibit functional responses mediated by bradykinin, such as intracellular calcium mobilization (38, 39). Phorbol esters are well known to alter phosphoinositide hydrolysis and calcium mobilization induced by agonists in various cell lines (2, 3, 13). We report that short term (5–90 min) TPA treatment of A549 cells attenuated the maximal increase in $[Ca^{2+}]_i$ induced by bradykinin, mediated by the kinin B_2 receptor. This reduction in bradykinin-evoked calcium mobilization by TPA was completely reversed with staurosporine, which is consistent with previous observations (13, 33). Treatment of A549 cells with TPA or staurosporine for 24 hr resulted in no effect on calcium mobilization (results not shown). These observations differ from previous reports, showing that pretreatment of cells with phorbol esters over a extended period of time (4) or with PKC inhibitors (33) caused a marked potentiation of agonist-induced phospholipase C activation in various cell lines. The lack of effect of PKC inhibition on basal activity may mean that PKC is not involved in regulating kinin B_2 receptor coupling to calcium mobilization in the A549 cells, except for acute activation of PKC by phorbol esters. These results in A549 cells differ from those in astrocytes (4), for example, which is probably related to differences in the cell types.

To evaluate the putative role of PKC-α in bradykinin B_2 receptor regulation by phorbol esters, we used an antisense oligonucleotide to selectively reduce PKC-α protein expression in A549 cells. A previously reported 20-mer phosphorothioate oligodeoxynucleotide (ISIS 3521) was shown to spe-

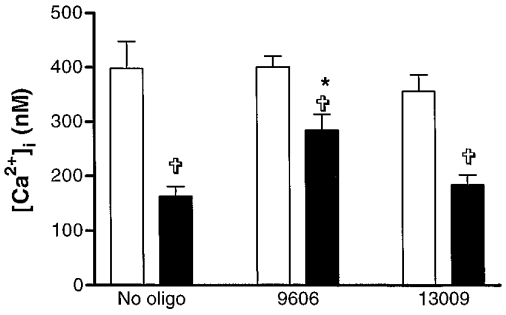

Fig. 7. Effect of oligonucleotides on TPA-induced reduction of bradykinin-evoked increases in peak $[Ca^{2+}]_i$ levels in A549 cells. Cells are harvested 72 hr after treatment with 150 nM of ISIS 9606 or ISIS 13009. Cells were then challenged with bradykinin alone (100 nM; *open bars*) or pretreated with TPA (500 nM; *solid bars*) 15 min before the addition of bradykinin. Values are mean ± standard error of 12 determinations. The effect of TPA (*filled columns*) was compared with cells not treated with TPA (*open columns*) using Student's *t* test (*open dagger*, $p < 0.01$). The oligonucleotides was found to have no effect of bradykinin-induced peak $[Ca^{2+}]_i$ levels (compare all the open bars) using one-way analysis of variance. *Filled bar for 9606*, significantly different from *no oligo* and *13009*. One-way analysis of variance was performed to assess the effects of oligonucleotides on the TPA treatment ($p < 0.01$). Dunnett's test was then applied to compare the effects of 9606 with no oligonucleotide treatment and 13009 (*, $p < 0.01$)

cifically reduce human PKC-α expression in tissue culture (22, 25). However, to further validate the mechanisms involved, the effects of ISIS 3521 were compared with those of a series of length-matched oligonucleotides that contain a increasing number of mismatches. The gradual increase in the number of base mismatches decreased the melting temperature of the oligonucleotides for the complementary sense strand and the potency of PKC-α mRNA reduction. The reduction in PKC-α mRNA induced by ISIS 3521 was reversible, and the expression of the mRNA approached control values after 72 hr (22). This resulted in only a temporary loss of PKC-α protein (22). Chemical modifications have been incorporated into the 2′-sugar position of the sequence, enhancing the oligonucleotide activity by increasing melting temperature (Table 1), affinity for the sense strand, and nuclease resistance (26). Although the modification did not increase the biological potency of the oligonucleotide (ISIS 3521, $EC_{50} \sim 80$ nM; ISIS 9606, $EC_{50} \sim 90$ nM), it dramatically increased the duration of action (22). ISIS 9606 reduced the concentration of PKC-α mRNA transcripts in a concentration-dependent fashion over a time period of 72 hr. Thus, the clear advantage of using modified oligodeoxynucleotides is the ability to reduce mRNA expression for longer periods of time, thus permitting reduction of proteins with long half-lives such as PKC-α. At 150 nM, ISIS 9606 reduced the immunoreactive PKC-α protein by 66.3 ± 2.5% 72 hr after treatment. However, higher concentrations of oligonucleotide and longer treatments (96 hr) did not lead to greater reductions in protein levels. There are a number of potential explanations for this. Because PKC-α protein has a half-life varying from 6.7 to >24 hr (34, 35), complete elimination of the protein is difficult in our experimental conditions. Another explanation would involve the association of PKC to cytoskeletal (36, 37) or nuclear proteins (38, 39), which could act as a reservoir for the enzyme and prevent its proteolysis. Alternatively, transfection of oligonucleotides is well known to result in uptake into only a fraction of the cells treated (40), and this would result in partial effects.

The partial reduction in PKC-α protein expression by ISIS 9606 had a significant effect on phorbol ester-induced reduction in calcium mobilization evoked by bradykinin in A549 cells. In contrast to the partial reversal of the phorbol ester effects induced by ISIS 9606, staurosporine, which is a nonspecific PKC inhibitor, completely reversed the effect. Given the partial reduction in PKC-α protein produced by ISIS 9606, we cannot determine whether the limited reversal of the phorbol ester effects is due to the fact that some PKC-α protein remained after treatment or that other PKC isozymes are involved in this process. It was previously suggested that PKC-δ participates in the regulation of the kinin B_2 receptor by phorbol esters in astrocytes (4). This could also contribute to the limited reversal of the effects of phorbol esters by ISIS 9606 in our model. Nevertheless, these results suggest that PKC-α plays a important role in phorbol ester-mediated receptor regulation of the bradykinin B_2 receptors in the A549 cells. These results constitute the first demonstration that PKC-α may regulate bradykinin-induced mobilization of calcium. Combined with the previous suggestion (4) that PKC-α is involved in the regulation of bradykinin-induced inositol metabolism, it is apparent that PKC-α plays a central regulatory role in signaling through kinin B_2 receptors.

In conclusion, our results demonstrate that antisense oligonucleotides can be used to specifically lower the expression of PKC-α mRNA and protein *in vitro.* Partial reduction in PKC-α protein has a major effect on phorbol ester-induced reduction of bradykinin-evoked calcium mobilization in A549 cells. Thus, we have demonstrated that PKC-α plays a role in phorbol ester-mediated regulation of the bradykinin B_2 receptors in the A549 cells. This is the first time that antisense oligonucleotides were used to determine which PKC isozyme is involved in the regulation of receptors coupled to phospholipase C in cells.

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