Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 11762–11766, November 1994
Pharmacology

Inhibition of protein kinase C-α expression in mice after systemic administration of phosphorothioate antisense oligodeoxynucleotides

NICHOLAS M. DEAN* AND ROBERT MCKAY

Department of Molecular Pharmacology, ISIS Pharmaceuticals, Carlsbad, CA 92008

Communicated by Pedro Cuatrecasas, July 19, 1994 (received for review February 17, 1994)

ABSTRACT **A 20-mer phosphorothioate oligodeoxynucleotide designed to hybridize to the AUG translation initiation codon of mRNA encoding murine protein kinase C-α (PKC-α) inhibits the expression of PKC-α both *in vitro* and *in vivo*. In mouse C127 mammary epithelial cells, the reduction in PKC-α mRNA expression was both dose and time dependent. The oligodeoxynucleotide exhibited an IC_{50} value of 100–200 nM and reduced PKC-α mRNA expression for up to 48 hr. This reduction was specific for PKC-α versus other PKC isozymes (δ, ε, and ζ) and completely dependent upon oligodeoxynucleotide sequence. When administered intraperitoneally in mice, the same oligodeoxynucleotide caused a dose-dependent, oligodeoxynucleotide sequence-dependent reduction of PKC-α mRNA in liver, with an IC_{50} value of 30–50 mg/kg of body weight. Inhibition of expression was 64 ± 11% after a single 50-mg/kg dose. The expression of PKC-δ, ε, and ζ mRNA was unaffected by this treatment. The oligodeoxynucleotide activity *in vivo* did not require the presence of cationic liposomes or any other delivery systems, although *in vitro*, the oligodeoxynucleotide required cationic liposomes for inhibition of PKC-α expression. This study demonstrates the utility of phosphorothioate oligodeoxynucleotides as specific inhibitors of gene expression *in vivo* after systemic administration.**

Oligonucleotides designed to hybridize to specific pre-mRNA or mRNA sequences (antisense oligonucleotides) have been shown to inhibit the expression of numerous viral and mammalian genes *in vitro* (1–3). Phosphorothioate oligodeoxynucleotides, in which one of the nonbridging oxygens in the backbone of DNA is replaced by sulfur, are one of the most used and best characterized class of oligodeoxynucleotides used as antisense agents (1, 2). The sulfur substitution dramatically increases nuclease stability, resulting in substantially improved pharmacokinetic properties compared with phosphodiester oligodeoxynucleotides (4, 5).

Despite the demonstration that appropriately designed phosphorothioate oligodeoxynucleotides can inhibit the expression of a large number of genes with high selectivity (6–8), skepticism about their utility remains (9). This derives from several factors. First, phosphorothioates are negatively charged and have shown wide variation in uptake by cells in tissue culture as a function of cell type, tissue culture conditions, and oligodeoxynucleotide sequence (10, 11). Second, studies in our laboratory have shown very clearly the differing sensitivity of sites in RNA molecules to inhibition by phosphorothioate oligodeoxynucleotides (6, 12–15). Thus, failure to perform careful dose–response studies in several cell lines with oligodeoxynucleotides designed to bind to a variety of sites in a target RNA molecule could result in conclusions that phosphorothioate oligodeoxynucleotides are inactive as antisense drugs or that their effects are due to nonspecific interactions.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Perhaps more importantly, recent studies in several species of animals with different phosphorothioate oligodeoxynucleotides have shown that they have attractive pharmacokinetic properties that are not predicted by *in vitro* cell uptake studies. They are rapidly and extensively absorbed from all parenteral sites of administration studied (16–18). Oligodeoxynucleotides are widely distributed to all peripheral tissues, with liver and kidney accumulating the most drug (16–19), and are slowly cleared by metabolism (16, 17, 19). Moreover, several laboratories have reported both local and systemic effects consistent with an antisense mechanism (20–22).

To better understand the *in vivo* utility of phosphorothioate oligodeoxynucleotides, we have designed oligodeoxynucleotides to inhibit mouse protein kinase C-α (PKC-α). The PKC family of enzymes consists of at least 10 closely related gene products which are involved in the propagation of signals initiated at the cell surface (23). PKC-α is one of the subspecies of PKC known as classical PKCs (23), which bind diacylglycerol and require Ca^{2+} for full activation. PKC is thought to be involved in inflammatory processes, immune responses, and cell proliferation; however, knowledge of specific functions of individual isozymes is still limited (23). We chose this target because it could be studied both in cultured cells and in mice. In addition, oligodeoxynucleotide specificity could be rigorously evaluated by comparing effects on PKC-α to effects on other PKC isotypes.

MATERIALS AND METHODS

Cell Culture. Murine C127 mammary epithelial cells and NIH 3T3 fibroblasts were obtained from the American Type Culture Collection. Murine bEND.3. endothelioma cells were obtained from Werner Risau, Max Planck Institute, Planegg-Martinsreid, Germany. Cell lines were grown in Dulbecco's modified Eagle's medium containing 1 g of glucose per liter (DMEM); (GIBCO/BRL) and 10% fetal bovine serum (FBS, HyClone) and routinely passaged when 90–95% confluent.

Oligonucleotide Synthesis and Sequences. Phosphorothioate oligodeoxynucleotides and 2′-*O*-methyl phosphorothioate oligonucleotides were synthesized as described (6, 23). All oligonucleotides were >85% full-length material when analyzed by electrophoresis in polyacrylamide gels. In addition, the relative amounts of phosphorothioate and phosphodiester linkages obtained by our synthesis were periodically checked by ^{31}P nuclear magnetic resonance spectroscopy (6). Phosphorothioate oligonucleotides were synthesized on a 0.5-mmol scale on a Milligen model 8800 DNA synthesizer using modified phosphoramidite chemistry with β-cyanoethoxyphosphoramidites (6). Oligonucleotides were depyrogenated

Abbreviations: DOPE, dioleoyl phosphatidylethanolamine; DOTMA, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride; FBS, fetal bovine serum; G3PDH, glycerol-3-phosphate dehydrogenase; PKC, protein kinase C.

*To whom reprint requests should be addressed.

by ultrafiltration, with endotoxin below detectable levels (commercial gel clotting methods; Associates of Cape Cod, Falmouth, MA). The sequence of the phosphorothioate oligodeoxynucleotide (ISIS 4189) designed to hybridize to murine PKC-α mRNA is 5′-CAGCCATGGTTCCCCCCAAC-3′ (which is complementary to positions 243–262 on the PKC-α mRNA (24). The 2′-*O*-methyl phosphorothioate oligonucleotide with the same sequence as this is ISIS 4999. The sequence of the scrambled control oligodeoxynucleotide (ISIS 4969) is 5′-CCAGTCACTCGCACCATCGC-3′. The sequence of the control oligodeoxynucleotide ISIS 1082 is 5′-GCCGAGGTCCATGTCGTACGC-3′.

Treatment of Cells with Oligonucleotides. C127, NIH 3T3, and bEND.3. cells were grown in T-75 flasks until 70–80% confluent. At this time, cells were washed twice in 10 ml of DMEM and 5 ml of DMEM containing *N*-[1-(2,3-dioleoyloxy)-propyl]-*N*,*N*,*N*-trimethylammonium chloride/dioleoyl phosphatidylethanolamine (DOTMA/DOPE) solution (Lipofectin, final concentration, 20 μg/ml; GIBCO/BRL) was added. Oligonucleotides were then added to the required concentration from a 10 μM stock solution and the two solutions were mixed by swirling of the dish. The cells were incubated at 37°C for 4 hr, washed once with DMEM/10% FBS to remove the DOTMA/DOPE solution, and then allowed to recover for the time indicated in the figure legends.

Treatment of Mice with Oligonucleotides. Female SK-1 hairless mice were housed three to a cage under conditions meeting or exceeding National Institutes of Health regulations (25). Oligodeoxynucleotides were administered in 0.9% NaCl (200 μl) i.p. at the dose and time indicated in the figure legends.

Measurement of PKC Isozyme mRNA Levels. Total mRNA was isolated from tissue culture cells by lysis in 4 M guanidinium isothiocyanate followed by cesium chloride gradient centrifugation (26). Total mRNA was isolated from various mouse organs by rapid homogenization of the tissue in 4 M guanidinium isothiocyanate followed by centrifugation over a cesium chloride gradient. RNA (20–40 μg) was resolved in 1.2% agarose gels containing 1.1% formaldehyde and transferred to nylon membranes. The blots were then hybridized with bovine PKC-α cDNA (American Type Culture Collection) or PKC-δ, PKC-ε, or PKC-ζ cDNA probes (obtained from Doriano Fabbro, CIBA–Geigy) as described (15). To obtain better sensitivity, we sometimes prepared ^{32}P-labeled PKC-α probe by PCR (27). In brief, a 1.3-kb bovine PKC-α fragment was used as the template in an asymmetric PCR with [α-^{32}P]dCTP. The antisense primer yielded a single product of 740 bases. Probe hybrized to mRNA transcripts was visualized and quantitated with a PhosphorImager (Molecular Dynamics). PKC-α mRNA is expressed as two transcripts, 8.5 and 4.0 kb. The blots were then stripped of radioactivity by boiling and reprobed with a ^{32}P-labeled glycerol-3-phosphate dehydrogenase (G3PDH) probe (Clontech) to confirm equal loading.

Immunoblotting of PKC Isozymes. C127 cells were treated with oligonucleotides, washed twice in cold phosphate-buffered saline and then extracted in 250 μl of lysis buffer [20 mM Tris, pH 7.4/1% (vol/vol) Triton X-100/5 mM EGTA/2 mM EDTA/2 mM dithiothreitol/50 mM sodium fluoride/10 mM sodium phosphate with leupeptin (2 μg/ml) and aprotinin (1 μg/ml)] at 4°C. The protein content of the various samples was determined with a Bio-Rad DC protein assay [based on the Lowry method (28)] using bovine serum albumin as standard. The expression of various PKC isozymes was then determined (15) by use of anti-PKC-α monoclonal antibody (1 μg/ml) (Upstate Biotechnology, Lake Placid, NY) or anti-PKC-δ, -ζ, or -ε polyclonal antibodies (1:2000) (GIBCO/BRL).

RESULTS

Twenty oligodeoxynucleotides designed to hybridize to multiple sites on the murine PKC-α mRNA (24) (including 5′ untranslated region, coding region, and 3′ untranslated region) were initially synthesized as 20-mer phosphorothioate oligodeoxynucleotides and tested for their ability to inhibit PKC-α mRNA expression in C127 murine mammary epithelial cells (data not shown). One oligodeoxynucleotide, ISIS 4189, which hybridizes to the AUG translation initiation codon, consistently inhibited PKC-α expression in C127 cells better than other oligodeoxynucleotides examined. At 400 nM, ISIS 4189 decreased the expression of both the 4-kb and the 8.5-kb PKC-α mRNA by >90% after 24 hr (Fig. 1*A*). A similar inhibition of PKC-α mRNA expression was also found in NIH 3T3 fibroblasts and bEND.3. endothelioma cells (Fig. 1*A*). We further characterized the inhibition in C127 cells and found it to be concentration dependent, with an IC_{50} value of 100–200 nM (Fig. 1*B*). The effects of ISIS 4189 on PKC-α

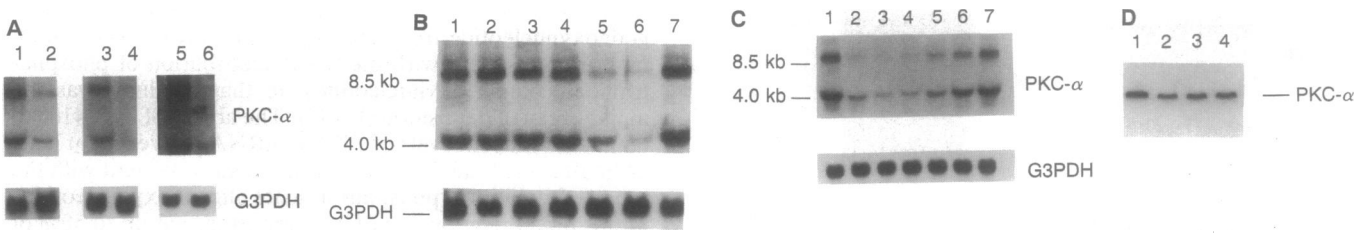

FIG. 1. Inhibition of PKC-α expression by oligonucleotides in cultured cells. (*A*) Effect of ISIS 4189 on PKC-α mRNA expression. C127 cells (lanes 1 and 2), NIH 3T3 cells (lanes 3 and 4), and bEND.3. cells (lanes 5 and 6) were treated with either 400 nM ISIS 4189 and DOTMA/DOPE (20 μg/ml) solution (lanes 2, 4, and 6) or just DOTMA/DOPE (20 μg/ml) solution (lanes 1, 3, and 5) for 4 hr. Cells were then washed with DMEM/10% FBS and allowed to recover for an additional 20 hr. Total RNA was then extracted and 20 μg was resolved in 1.2% agarose gels and transferred to nylon membranes as described in *Materials and Methods*. The blots were probed with a ^{32}P-radiolabeled PKC-α cDNA probe (*Upper*) and then stripped and reprobed with a ^{32}P-radiolabeled G3PDH probe to confirm equal RNA loading (*Lower*). (*B*) Dose response for the reduction in PKC-α mRNA by ISIS 4189. C127 cells were treated with oligonucleotide and DOTMA/DOPE (20 μg/ml) for 4 hr. Cells were then washed once with DMEM/10% FBS and allowed to recover for an additional 20 hr. PKC-α mRNA expression was determined by Northern blotting as in *A*. Lane 1, ISIS 4969 (scrambled control) (300 nM); lane 2, phosphate-buffered saline; lanes 3–6, ISIS 4189 (50, 100, 200, and 300 nM); lane 7, ISIS 4999 (2′-*O*-methyl phosphorothioate 4189) (300 nM). (*C*) Kinetic analysis of the reduction in PKC-α mRNA by ISIS 4189. C127 cells were extracted at zero time (lane 1) or treated with oligodeoxynucleotide (400 nM) and DOTMA/DOPE (20 μg/ml) solution for 4 hr. Cells were then washed once with DMEM/10% FBS and allowed to recover for 0 hr (lane 2), 8 hr (lanes 3), 20 hr (lane 4), 44 hr (lane 5) or 68 hr (lane 6 and 7). Oligodeoxynucleotide treatments were with ISIS 4189 (lanes 2–6) or ISIS 4969 (lane 7). PKC-α mRNA expression was determined by Northern blotting as in *A*. (*D*) Kinetic analysis of the reduction in PKC-α protein by ISIS 4189. C127 cell protein was extracted at zero time (lane 1), or cells were treated with ISIS 4189 (400 nM) and DOTMA/DOPE (20 μg/ml) for 4 hr. Cells were then washed once with DMEM/10% FBS and allowed to recover for 20 hr (lane 2), 44 hr (lane 3), or 68 hr (lane 4). Cell protein was then extracted and PKC-α protein expression was determined by immunoblotting.

transcripts were not observed with a scrambled-sequence control phosphorothioate oligodeoxynucleotide (ISIS 4969) or another control phosphorothioate oligodeoxynucleotide (ISIS 1082) (Fig. 1*B* and Fig. 2). None of the oligodeoxynucleotides tested affected G3PDH mRNA levels, demonstrating selectivity for the targeted mRNA (Fig. 1). We also tested a 2′-*O*-methyl phosphorothioate analog of ISIS 4189 (ISIS 4999) for its ability to decrease PKC-α mRNA. Although 2′-*O*-methyl phosphorothioate oligonucleotides bind to complementary RNA with a higher affinity than the corresponding phosphorothioate oligodeoxynucleotides, they do not support RNase H-mediated cleavage of the hybridized RNA (29). ISIS 4999 was completely ineffective at decreasing PKC-α mRNA (Fig. 1*B*), strongly suggesting that the decrease in mRNA seen with ISIS 4189 was mediated by RNase H.

We next determined the kinetics of the mRNA decrease and found that at the highest concentration of oligodeoxynucleotide (400 nM), 60–70% of the maximal decrease in PKC-α mRNA had occurred within 4 hr (Fig. 1*C*). The greatest reduction in PKC-α mRNA occurred 12–24 hr after oligodeoxynucleotide addition. The reduction in PKC-α mRNA was reversible; 72 hr after oligodeoxynucleotide treatment, PKC-α mRNA levels had returned to about 50% of control (Fig. 1*C*). This rebound is most likely the result of oligodeoxynucleotide degradation by intracellular nucleases resulting in lower intracellular levels of oligodeoxynucleotide after extended periods of time. PKC-α protein has been reported to have a relatively long half-life (from 6.7 to >24 hr) (30–32). This requires that the levels of PKC-α mRNA are inhibited for extended periods of time with oligodeoxynucleotides to achieve a reduction in steady-state levels of protein.

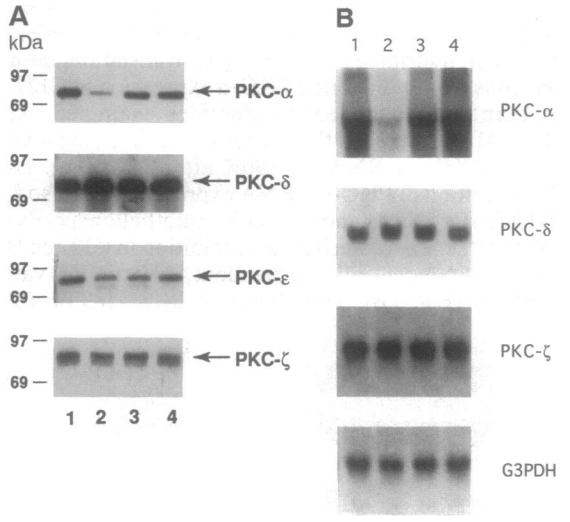

FIG. 2. Isozyme-specific reduction in PKC expression by ISIS 4189. (*A*) Protein. C127 cells were treated with oligonucleotide (400 nM) and DOTMA/DOPE (20 μg/ml) for 4 hr. Cells were then washed once with DMEM/10% FBS and allowed to recover for 44 hr. The cells were then washed once in serum-free medium and treated with oligonucleotide (200 nM) and DOTMA/DOPE (20 μg/ml) for an additional 4 hr. Cells were then washed once with DMEM/10% FBS and allowed to recover for 20 hr. At this time cell proteins were extracted and immunoreactive PKC-α, -δ, -ε, or -ζ was determined by immunoblotting. Molecular mass standards are shown at left and the PKC isozymes are indicated by arrows at right. Treatments: lane 1, saline control; lane 2, ISIS 4189; lane 3, ISIS 4969; lane 4, ISIS 4999. (*B*) mRNA. C127 cells were treated with oligodeoxynucleotide (400 nM) and DOTMA/DOPE (20 μg/ml) for 4 hr. Cells were then washed once with DMEM/10% FBS and allowed to recover for 20 hr. Total RNA was extracted and 20 μg was resolved in four identical gels. Treatments: lane 1, saline control; lane 2, ISIS 4189; lane 3, ISIS 4969; lane 4, ISIS 1082. Blots were probed with ^{32}P-labeled PKC-α, -δ, or -ζ or G3PDH cDNA probes.

A single treatment of C127 cells with 400 nM oligodeoxynucleotide was found to decrease PKC-α protein by 50% after 24 and 48 hr, with levels recovering to 70–80% of control after 72 hr (Fig. 1*D*). This recovery in PKC-α protein is most likely the result of the recovery in PKC-α mRNA (Fig. 1*C*) and demonstrates that a single application of ISIS 4189 is not sufficient to decrease PKC-α protein to levels below about 50%. To maintain decreased levels of PKC-α mRNA for extended periods of time, C127 cells were treated with repeat applications of oligodeoxynucleotide in the presence of DOTMA/DOPE. An initial maximal concentration of 400 nM followed 2 days later by 200 nM gave the best prolonged PKC-α mRNA reduction. Using this approach, we successfully decreased steady-state levels of PKC-α protein by 70–80% after 72 hr (Fig. 2*A*).

Northern blotting and immunoblotting of C127 cells with PKC isozyme-specific cDNA probes and antisera revealed that in addition to PKC-α these cells express PKC-δ, -ε, and -ζ. Under conditions where ISIS 4189 decreases PKC-α protein by 70–80%, levels of PKC-δ, -ε, and -ζ were unchanged (Fig. 2*A*). PKC-α protein was unaffected by treatment of cells with ISIS 4969 (a scrambled control) and ISIS 4999 (2′-*O*-methyl phosphorothioate analog of 4189) (Fig. 2*A*). In addition, the antisense oligodeoxynucleotide-mediated decrease in PKC-α mRNA expression was isozyme specific. PKC-δ and -ζ mRNA levels were unchanged by treatment of C127 cells with a concentration of ISIS 4189 (400 nM) that decreased PKC-α mRNA by >90% (Fig. 2*B*).

We have extended these *in vitro* studies to determine the ability of oligodeoxynucleotides to inhibit gene expression after systemic administration in mice. ISIS 4189 or a non-complementary control oligonucleotide, ISIS 1082, was administered i.p. at doses of 1, 10, or 100 mg/kg per day for 7 days. The expression of PKC-α or a constitutively expressed gene product, G3PDH, was examined in various tissues 24 hr after administration of the last dose. PKC-α mRNA in liver was decreased in a dose-dependent manner. Treatment of mice with ISIS 4189 at 100 mg/kg per day for 7 days resulted in a 90% reduction in PKC-α mRNA (Fig. 3*A*), whereas 10 mg/kg per day resulted in a 20% reduction (Fig. 3*B*). In a separate study, a dose of 50 mg/kg decreased PKC-α mRNA by 68% (Fig. 3*B*). The control oligodeoxynucleotide ISIS 1082 failed to significantly affect PKC-α mRNA. Neither ISIS 4189 nor the control oligodeoxynucleotide affected the level of G3PDH expression. PKC-α mRNA in lung, spleen, kidney, and skin was not reproducibly decreased by any oligodeoxynucleotide treatment (data not shown). These findings are consistent with the tissue distribution of phosphorothioate oligodeoxynucleotides, in that the liver was the major organ of deposition (16–19). The ability of ISIS 4189 to maintain reduced levels of PKC-α mRNA in liver for at least 24 hr after the final dose was given is also consistent with the reported stability of phosphorothioate oligodeoxynucleotides in this organ. Agrawal *et al.* (18) reported that up to 50% of a 20-mer phosphorothioate oligodeoxynucleotide remained intact as full-length material in liver up to 48 hr after administration.

The kinetics and specificity of the oligodeoxynucleotide-mediated reduction in PKC-α mRNA in mouse liver were also examined. Phosphorothioate oligodeoxynucleotide (either ISIS 4189 or ISIS 1082) was administered i.p. at a dose of 50 mg/kg per day for 1, 4, or 7 days and PKC mRNA expression in liver was determined by Northern blotting 24 hr after the final injection. Treatment of mice for 7 days with ISIS 4189 reduced expression of PKC-α by 68 ± 11% (n = 3) (Fig. 3 *B* and *C*). In addition, only a single treatment with ISIS 4189 was sufficient to decrease PKC-α mRNA by 64 ± 11% (n = 3) (Fig. 3*C* and data not shown). The same mRNA samples were analyzed by Northern blotting for expression of other PKC isozymes. In addition to PKC-α mRNA, we

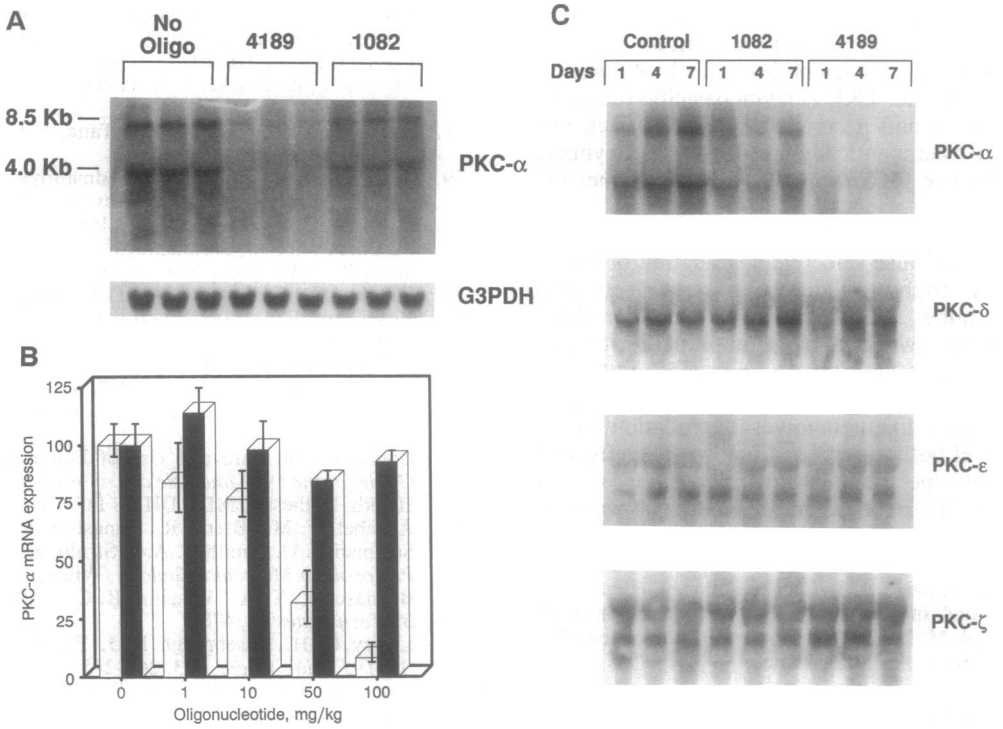

FIG. 3. Effect of oligodeoxynucleotides on expression of PKC-α in mouse liver. (*A*) Effect of ISIS 4189 and 1082 on expression of PKC-α mRNA. SK-1 hairless mice (18) were given oligodeoxynucleotide (100 mg/kg) i.p., in a volume of 200 μl in saline every day for 7 days (three animals per group). On day 8, animals were sacrificed and samples of liver were removed. Total RNA was isolated and 40 μg was resolved in a 1% agarose gels. RNA was transferred to nylon membrane and probed with a ^{32}P-labeled PKC-α-specific probe generated by PCR (a 1.3-kb bovine PKC-α fragment was used as the template in an asymmetric PCR with [α-^{32}P]dCTP). The antisense primer yielded a single-stranded product of 740 bases. Each lane shows RNA obtained from an individual animal. The blot was then stripped and reprobed with a ^{32}P-labeled G3PDH probe to demonstrate equal loading. (*B*) Dose response for the inhibition of PKC-α mRNA expression in mouse liver by ISIS 4189. Mice were treated as described in *A* with ISIS 4189 (open bars) or 1082 (black bars) at 100, 10, or 1 mg/kg of body weight in one experiment and 50 mg/kg in a second experiment. PKC-α expression in liver was determined by Northern blotting and is expressed relative to G3PDH (n = 3). (*C*) Time course and specificity for the effects of oligonucleotides on PKC isozyme expression in mouse liver. Mice were treated as described in *A* with ISIS (4189 or 1082) at 50 mg/kg for 1, 4, or 7 days. PKC-α, -δ, -ε, and -ζ mRNA expression was determined by Northern blotting. All four lanes contained mRNA from the same individual.

detected mRNA for PKC-δ, -ε, and -ζ. Expression of the other isozymes was not reduced by treatment with either ISIS 4189 or ISIS 1082 at any time following oligonucleotide administration (Fig. 3*C*). Because of the low levels of expression of PKC-α protein in liver and variability in Western blotting, we have not attempted to quantitate changes in protein levels in the liver.

DISCUSSION

Many studies have reported biological effects of phosphorothioate oligodeoxynucleotides *in vivo*. For example, a reduction in tumor growth has been reported for transplanted tumors in nude mice and syngeneic mice treated with oligodeoxynucleotides targeting c-myb and the p65 and p50 subunits of NF-κB (8, 20, 33). These effects were accompanied by a reduction in expression of p65 in the tumor in one of the studies, suggesting that the oligodeoxynucleotide inhibition of tumor growth was mediated through a true antisense mechanism (20). To date, however, the demonstration of a reduction in normal, constitutively expressed host mRNA *in vivo* by oligodeoxynucleotides has been limited to localized administration of the oligodeoxynucleotides or with oligodeoxynucleotides formulated with liposomes (21, 34). Our studies have extended these findings on the utility of phosphorothioate oligodeoxynucleotides as inhibitors of gene expression *in vivo*. We show that the localized application of oligodeoxynucleotides to a target organ is not necessarily required to obtain a reduction in the targeted mRNA and that systemic oligodeoxynucleotide delivery may be all that is required to obtain inhibition in certain organs. We have also determined the doses at which oligodeoxynucleotides inhibit gene expression *in vivo* and demonstrated that this effect is relatively long lasting.

Oligodeoxynucleotide activity in tissue culture can be enhanced by as much as 1000-fold by preparing the oligodeoxynucleotide with cationic liposome formulations such as DOTMA/DOPE (35). However, our findings demonstrate that a requirement for cationic liposomes *in vitro* to show oligodeoxynucleotide activity does not preclude systemic activities *in vivo* in the absence of cationic lipids or other delivery systems. A recent report also suggests that uptake and expression of plasmid DNA in mice after intramuscular injection may not require liposomal encapsulation of the DNA (36).

Considerable efforts have been made to develop inhibitors of PKC, and some relatively specific compounds have been identified (see refs. 37 and 38). To our knowledge, however, isozyme-specific inhibitors of PKC have not previously been found. One approach which has been used to investigate specific functions of PKC isozymes includes the expression of antisense cDNA constructs in tissue culture (39, 40). These studies have demonstrated potential roles for PKC-α in regulating P-glycoprotein function and phorbol ester-mediated arachidonate release (39, 40). Homologous recombination has been used to study PKC isozyme function *in vivo*. Mice which are PKC-γ deficient have been developed and the behavioral changes seen in these animals suggest a role for PKC-γ in hippocampal long-term potentiation and spatial learning (41, 42). Our results demonstrate that an-

tisense oligodeoxynucleotides can also be used to achieve isozyme-selective inhibition, both *in vivo* and *in vitro*. The use of antisense oligodeoxynucleotides will allow studies to evaluate PKC-α and other PKC isozyme-specific functions, both in tissue culture and in animals. This approach may ultimately lead to therapeutically useful oligodeoxynucleotides targeting human PKC-α or other PKC isozymes (37, 38, 43).

In conclusion, a phosphorothioate oligodeoxynucleotide (ISIS 4189) shown to cause selective degradation of PKC-α mRNA *in vitro* was found to induce the same effects when administered to mice. We demonstrated a clear dose–response relationship with the ID_{50} for reduction of liver PKC-α mRNA being <50 mg/kg. The mechanism of action of the oligodeoxynucleotide is consistent with an antisense mechanism and most likely involves degradation of the mRNA by RNase H both *in vitro* and *in vivo*. The effects of ISIS 4189 are highly specific both *in vitro* and *in vivo*, as no effects on either G3PDH or other PKC isotypes were observed.

We gratefully acknowledge S. T. Crooke and C. F. Bennett for helpful discussions and suggestions during the preparation of the manuscript. We thank M. Zounes for synthesis of the oligonucleotides and B. Austin for technical assistance.

1. Crooke, S. T. & Lebleu, B., eds. (1993) *Antisense Research and Applications* (CRC, Boca Raton, FL).
2. Stein, C. A. & Cheng, Y.-C. (1993) *Science* **261**, 1004–1012.
3. Crooke, S. T. (1992) *Annu. Rev. Pharmacol. Toxicol.* **32**, 329–376.
4. Akhtar, S., Kole, R. & Juliano, R. L. (1991) *Life Sci.* **49**, 1793–1801.
5. Hoke, G. D., Draper, K., Gonzalez, C., Driver, V. B., Zounes, M. C. & Ecker, D. J. (1991) *Nucleic Acids Res.* **19**, 5743–5748.
6. Chiang, M.-Y., Chan, H., Zounes, M. A., Freier, S. M. & Bennett, C. F. (1991) *J. Biol. Chem.* **266**, 18162–18171.
7. Monia, B. P., Johnston, J. F., Ecker, D. J., Zounes, M. A., Lima, W. F. & Freier, S. M. (1992) *J. Biol. Chem.* **267**, 19954–19962.
8. Kitajima, I., Shinohara, T., Bilakovics, J., Brown, D. A., Xu, X. & Nerenberg, M. (1992) *Science* **258**, 1792–1795.
9. Wagner, R. W., Matteucci, M. D., Lewis, J. G., Gutierrez, A. J., Moulds, C. & Froehler, B. C. (1993) *Science* **260**, 1510–1513.
10. Crooke, R. M. (1991) *Anti-Cancer Drug Design* **6**, 609–646.
11. Gao, W.-Y., Storm, C., Egan, W. & Cheng, Y.-C. (1993) *Mol. Pharmacol.* **43**, 45–50.
12. Cowsert, L. M., Fox, M. C., Zon, G. & Mirabelli, C. K. (1993) *Antimicrob. Agents Chemother.* **37**, 171–177.
13. Azad, R. F., Driver, V. B., Tanaka, K., Crooke, R. M. & Anderson, K. P. (1993) *Antimicrob. Agents Chemother.* **37**, 1945–1954.
14. Bennett, C. F., Condon, T. P., Grimm, S., Chan, H. & Chiang, M.-Y. (1994) *J. Immunol.* **152**, 3530–3540.
15. Dean, N. M., McKay, R., Condon, T. P. & Bennett, C. F. (1994) *J. Biol. Chem.* **269**, 16416–16424.
16. Cossum, P. A., Sasmor, H., Dillinger, D., Truong, L., Cummins, Owens, S. R., Markham, P. M., Shea, J. P. & Crooke, S. (1993) *J. Pharmacol. Exp. Ther.* **267**, 1181–1190.
17. Cossum, P. A., Truong, L., Owens, S. R., Markham, P. M., Shea, J. P. & Crooke, S. T. (1994) *J. Pharmacol. Exp. Ther.* **269**, 89–94.
18. Agrawal, S., Temsamani, J. & Tang, J. Y. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7595–7599.
19. Temsamani, J., Tang, J. Y., Padmapriya, A., Kubert, M. & Agrawal, S. (1993) *Antisense Res. Dev.* **3**, 277–284.
20. Higgins, K. A., Perez, J. P., Coleman, T. A., Dorshkind, K., McComas, W. A., Sarmiento, U. M., Rosen, C. A. & Narayanan, R. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 9901–9905.
21. Simons, M., Edelman, E. R., DeKeyser, J.-L., Langer, R. & Rosenberg, R. D. (1992) *Nature (London)* **359**, 67–70.
22. Wahlestedt, C., Pich, E. M., Koob, G. F., Yee, F. & Heilig, M. (1993) *Science* **259**, 528–531.
23. Nishizuka, Y. (1993) *Science* **258**, 607–614.
24. Rose-John, R. S., Dietrich, A. & Marks, F. (1988) *Gene* **74**, 465–471.
25. Committee on Care and Use of Laboratory Animals (1985) *Guide for the Care and Use of Laboratory Animals* (Natl. Inst. Health, Bethesda, MD), DHHS Publ. No. 85-23.
26. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds. (1993) *Current Protocols in Molecular Biology* (Wiley, New York).
27. Bednasczsk, T. A., Wiggins, R. C. & Konat, G. W. (1991) *BioTechniques* **10**, 478.
28. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
29. Inoue, H., Hayase, Y., Iwai, S. & Ohtsuka, E. (1987) *FEBS Lett.* **215**, 327–330.
30. Ballester, R. & Rosen, O. (1985) *J. Biol. Chem.* **260**, 15194–15199.
31. Borner, C., Eppenberger, U., Wyss, R. & Fabbro, D. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2110–2114.
32. Young, S., Parker, P., Ullrich, A. & Stabel, S. (1987) *Biochem. J.* **244**, 775–779.
33. Ratajczak, M. Z., Kant, J. A., Luger, S. M., Hijiya, N., Zhang, J., Zon, G. & Gewirtz, A. M. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 11823–11827.
34. Morishita, R., Gibbons, G. H., Ellison, K. E., Nakajima, M., Zhang, L., Kaneda, Y., Ogihara, T. & Dzau, V. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8474–8478.
35. Bennett, C. F., Chiang, M.-Y., Chan, H., Shoemaker, J. E. & Mirabelli, C. K. (1992) *Mol. Pharmacol.* **41**, 1023–1033.
36. Ulmer, J. B., Donnelly, J. J., Parker, S. E., Rhodes, G. H., Felgner, P. L., Dwarki, V. J., Gromkowski, S. H., Deck, R. R., Dewitt, C. M., Friedman, A., Hawe, L. A., Leander, K. R., Martinez, D., Perry, H. C., Shiver, J. W., Montgomery, D. L. & Liu, M. A. (1993) *Science* **259**, 1745–1748.
37. Gescher, A. & Dale, I. L. (1989) *Anti-Cancer Drug Design* **4**, 93–105.
38. Wilkinson, S. E. & Hallam, T. J. (1994) *Trends Pharmacol. Sci.* **15**, 53–57.
39. Ahmad, S. & Glazer, R. I. (1993) *Mol. Pharmacol.* **43**, 858–862.
40. Godson, C., Bell, K. S. & Insel, P. (1993) *J. Biol. Chem.* **268**, 11946–11950.
41. Abeliovich, A., Chen, C., Goa, Y., Silva, A. J., Stevens, C. F. & Tonegawa, S. (1993) *Cell* **75**, 1253–1262.
42. Abeliovich, A., Paylor, R., Chen, C., Kim, J. J., Wehner, J. M. & Tonegawa, S. (1993) *Cell* **75**, 1263–1271.
43. Schwartz, G. K., Jiang, J., Kelsen, D. & Albino, A. P. (1993) *J. Natl. Cancer Inst.* **85**, 402–407.