An in vivo adenoviral gene delivery system was utilized to assess the effect of overexpressing protein kinase C (PKC-ζ) on rat skeletal muscle glucose transport activity. Female lean Zucker rats were injected with adenoviral/human PKC-ζ (hPKC-ζ) and adenoviral/LacZ in opposing tibialis anterior muscles. One week subsequent to adenoviral/gene delivery rats were subjected to hind limb perfusion. The hPKC-ζ protein was expressed at the same level (fast-twitch white) or at ~80% of the level (fast-twitch red) of endogenous PKC-ζ, thus approximately doubling the amount of PKC-ζ in tibialis anterior. Basal glucose transport activity was elevated -2.4-fold and 2-fold, respectively, in fast-twitch white and red hPKC-ζ muscle relative to control. Submaximal insulin-stimulated glucose transport activity, corrected for basal transport, was -90 and 40% over control values, respectively, in fast-twitch white and red hPKC-ζ muscle. The enhancement of glucose transport activity in muscle expressing hPKC-ζ occurred in the absence of any change in GLUT1 or GLUT4 protein levels, suggesting a redistribution of existing transporters to the cell surface. These results demonstrate that an adenoviral vector can be used to deliver expressible hPKC-ζ to adult rat skeletal muscle in vivo and also affirm a role for PKC-ζ in the regulation of glucose transport activity.

Elucidation of the specific intermediates involved in the metabolic arm of the insulin signaling cascade initiated by insulin binding to its receptor and culminating in GLUT4 translocation to the cell surface has been an area of intense investigation. To date, several key regulatory proteins and their indispensability in propagating the insulin signal, such as the insulin receptor, insulin receptor substrate proteins (IRS), and phosphatidylinositol 3-kinase (PI-3-K), have been identified and described (1–6). Downstream of PI-3-K, however, the path is less clear. The majority of evidence currently compiled implicates the protein kinase Akt/PKB as a primary target of inositol 3-phosphates, and associated regulatory proteins such as PDK-1, supporting its role as an insulin signaling intermediate (7–12). The existence of parallel or branched paths, however, at this point in the insulin signaling cascade have been suggested by the findings that inositol 3-phosphates and PDK-1 also activate a member of the atypical protein kinase C (PKC) family, PKC-ζ (11, 13).

PKC involvement in the stimulation of metabolism by insulin has been speculated on and investigated for many years, yet overall the evidence for or against a role for PKC in insulin signaling can best be described as equivocal (14). This ambiguity may stem, in part, from the diversity within the extensive PKC family that has been identified thus far. Normal insulin-stimulated glucose transport in 3T3-L1 cells despite down-regulation of conventional and novel PKC isoforms using pharmacological esters suggests that these isoforms are not necessary for insulin-induced activation of glucose transport (15–17). Alternatively, atypical PKC isoforms, such as PKC-ζ, are not diacylglycerol-sensitive and, as mentioned above, are activated by a well-established arm of the insulin signaling pathway, products of PI-3-K (11, 13). Interestingly, it has been demonstrated that PKC-ζ is activated by insulin and that overexpression of wild-type PKC-ζ increased, whereas overexpression of a dominant-negative PKC-ζ decreased, basal and insulin-stimulated glucose transport in 3T3-L1 cells (18). Likewise, stable and transient expression of a kinase-inactive PKC-ζ was shown to inhibit both basal and insulin-stimulated glucose transport in L6 myotubes (19). Collectively, these results suggest a potential role for PKC-ζ downstream of PI-3-K in the insulin signaling pathway for the stimulation of glucose transport.

To examine the potential ability of exogenously administered PKC-ζ to stimulate glucose transport activity in the primary insulin-responsive tissue, skeletal muscle, we expressed recombinant human PKC-ζ (hPKC-ζ) in rat tibialis anterior muscle using a novel in vivo adenoviral gene transfer system. Unlike classic transgenic studies, this approach affords several advantages including the following. Gene delivery and subsequent protein expression occurs in adult animals, and thus developmental compensatory responses are less likely to dictate the observed physiological outcome. Also, localization of gene delivery and protein expression within a specific muscle allows experimental and control genes to be delivered to the same animal, resulting in a highly controlled experimental paradigm. Finally, the ability to administer the adenoviral/gene construct to animals of various backgrounds or disease states eliminates the need for costly and time consuming breeding and backcrossing.

Application of this in vivo adenoviral/gene transfer system and subsequent assessment of physiological responses using the hind limb perfusion technique revealed that expression of hPKC-ζ in skeletal muscle of normal lean rats results in enhanced rates of both basal and submaximal insulin-stimulated glucose transport activity.

**MATERIALS AND METHODS**

*Generation of Recombinant Adenovirus—Recombinant adenovirus expressing β-gal was generated by homologous recombination between*
A recombinant adenovirus expressing hPKC-ζ was constructed as follows. The hPKC-ζ cDNA was isolated from hPKC-ζ Bluescript (22) on an XbaI to EcoRI fragment and first inserted between the XbaI and EcoRI sites of the cloning vector pOK12 (23). The hPKC-ζ cDNA was then excised as a 9-kilobase fragment and ligated into the BamHI site of pCA13 (Microbix Biosystems Inc. Ontario, Canada), thus generating the transfer plasmid pCA13/ζ. Using materials and protocols supplied by Microbix Biosystems Inc., recombination of pCA13/ζ and pBHG10 yielded the adenovirus Ad-hPKC-ζ.

Amplification and Purification/Concentration of Recombinant Adenoviruses—Amplification of recombinant virus was performed in adherent 293 cells. Purification and concentration of the virus was achieved using cesium chloride ultracentrifugation. The resulting virus was dialyzed into phosphate-buffered saline (with calcium and magnesium) plus 10% glycerol. Viral titers were determined using 100-mm dishes of adherent 293 cells overlaid with 0.6% agarose.

Animals and Immunosuppressive Therapy—Female lean Zucker rats (Fa/7) were obtained from Charles River Laboratories (Natick, MA) at 12 weeks of age. All animal procedures were reviewed and approved by the Institutional Animal Care Committee (IACUC) of the University of California, Los Angeles. Rats were maintained in an environmentally controlled facility with a 12-h light/12-h dark cycle and provided free access to food and water. Two days prior to adenoviral/gene injection, and on all days subsequent, rats received a 5 mg/kg subcutaneous injection of the immunosuppressive agent FK506 (Briegl, Burlington, MA) or saline. Viral titers were determined using 100-mm dishes of adherent 293 cells, and protein content of the homogenates was determined with a Bio-Rad protein assay (Bio-Rad, Hercules, CA). For Western blotting, membranes for subsequent detection with specific polyclonal antisera were blotted and probed with the horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech). The membrane was developed using an enhanced chemiluminescence (ECL-Plus; Amersham Pharmacia Biotech) and a Stork phosphoimager (Molecular Dynamics, Inc., Sunnyvale, CA).

Expression/activity of β-gal in muscle homogenates was determined by incubating 50 μl of each muscle homogenate with 100 μl of the chromogenic substrate X-gal (1 mg/ml) for 18 h. Statistical Analysis—All data are expressed as means ± S.E. The data were subjected to analysis of variance with significant differences between means determined using Fisher’s protected least significant difference post hoc analysis. Significance was set at the p < 0.05 level.

RESULTS AND DISCUSSION

Previous work with transgenic animals demonstrated that single gene manipulations can significantly alter normal and/or disease (diabetic) phenotypes (25–27). The application of these genetic manipulations was of limited practical implication, however, as a result of the technical aspects of gene insertion at the pro-nuclear stage of development. Such early manipulation also raises the question of developmental compensatory alterations leading to the observed adult transgenic phenotype. To circumvent such confounding factors and to investigate the practicality of acutely altering genetic expression in adult animals, we developed/defined a system for delivering genes of interest to a primary target of insulin action and a key regulator of glucose homeostasis, skeletal muscle. By combining the intramuscular adenoviral/gene injections with subsequent hind limb perfusion experiments, we were able to determine whether transcription of our gene of interest (hPKC-ζ) resulted in expression of a functional protein and, furthermore, determine whether the expression of that protein is physiologically relevant.

As shown in Fig. 1, lanes 2 and 4, intramuscular injection of the adenoviral/hPKC-ζ construct into tibialis anterior muscle resulted in the appearance of a second immunoreactive PKC-ζ band of slightly greater molecular weight than the endogenous band. We have observed that this migratory difference, between endogenous and exogenous PKC-ζ, on SDS-PAGE is species-related. Quantitative analysis revealed that hPKC-ζ expression equaled endogenous PKC-ζ expression in fast-twitch white muscle, whereas in fast-twitch red muscle hPKC-ζ was expressed at ~80% of endogenous levels (p = 0.01). There were no apparent systemic effects of the local injections as expression of hPKC-ζ (Fig. 1) and expression/activity of β-gal (data not shown) were noted only in those muscles directly injected.

Upon establishment of hPKC-ζ expression, attention was turned toward the metabolic consequences. Fig. 2 reveals glucose transport/activity was significantly modified during hind limb perfusion. In fast-twitch white muscle, hPKC-ζ expression enhanced basal glucose transport 3.4-fold above the β-gal control. Similarly, basal glucagon transport was elevated approximately 2-fold above control in fast-twitch red muscle expressing hPKC-ζ. It may be speculated that the difference between fiber types, with regard to magnitude of increase in transport activity, reflects the subtle disparity noted in the level of hPKC-ζ expression. The expression of β-gal in tibialis anterior muscle (fast-twitch

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FIG. 1. Representative autoradiographic samples of fast-twitch white (lanes 1 and 2) and red (lanes 3 and 4) tibialis anterior muscle homogenates immunoblotted for PKC-ζ protein. Lane 1, adenoviral/LacZ-injected white tibialis anterior (50 μg of protein); lane 2, adenoviral/hPKC-ζ-injected white tibialis anterior (50 μg of protein); lane 3, adenoviral/LacZ-injected red tibialis anterior (50 μg of protein); lane 4, adenoviral/hPKC-ζ-injected red tibialis anterior (50 μg of protein).
viral/Lac-Z injections and subsequent highly controlled nature of these experiments, in which adeno-
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