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Arginine-Specific ADP-Ribosyltransferase on the Surface of Gizzard Smooth Muscle Cells and the Involvement of Phosphatidylinositol 3-Kinase in Maintaining the Activity of This Transferase[1]

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An arginine-specific ADP-ribosyltransferase activity was detected in chicken gizzard smooth muscle, and the specific activity is highest in the membrane fraction. This transferase is released from the membrane fraction by phosphatidylinositol-specific phospholipase C (PI-PLC), suggesting that it is a glycosylphosphatidylinositol (GPI)-anchored protein. When primary cultured gizzard smooth muscle cells (SMCs) were incubated with [adenylate-^{32}P]NAD, several proteins were labeled. The labeling was inhibited by preincubation of the cells with PI-PLC, or by the addition of L-arginine to the reaction, and was sensitive to hydroxylamine treatment. The activity of the transferase was maintained in differentiated SMCs cultured with insulin, but was dramatically decreased concomitantly with cell dedifferentiation induced by serum or a specific PI3-kinase inhibitor, LY294002. These results indicate that the GPI-anchored arginine-specific ADP-ribosyltransferase is expressed on the surface of differentiated SMCs and can modify several cell surface proteins. Our results also suggest that PI3-kinase is involved in the regulation of transferase activity during differentiation.

Key words: arginine-specific ADP-ribosyltransferase, gizzard, GPI-anchor, PI3-kinase, smooth muscle cell.

Arginine-specific ADP-ribosylation is a post-translational modification in which the ADP-ribose moiety of NAD is transferred to arginine residues on target proteins by arginine-specific ADP-ribosyltransferase. Cholera toxin ADP-ribosylates an arginine residue in the Gsα, resulting in the inhibition of GTPase activity (*1*). In the photosynthetic bacterium *Rhodospirillum rubrum*, a nitrogenase is inactivated by ADP-ribosylation of a specific arginine residue (*2*) and is restored to its original activity when the ADP-ribose group is removed by an ADP-ribosylhydrolase (*3*). These results indicate that arginine-specific ADP-ribosylation is involved in the regulation of protein function.

In eucaryotes, an arginine-specific ADP-ribosyltransferase activity has been detected in striated muscles including rabbit skeletal (*4, 5*) and rat cardiac muscles (*6*). The enzyme is expressed on the surface of differentiated skeletal muscle cells *via* a glycosylphosphatidylinositol (GPI)-anchor (*7*), and it can ADP-ribosylate the extracellular domain of the adhesion molecule integrin α7 on cells (*7*). In the presence of an inhibitor of arginine-specific ADP-ribosyltransferase, differentiation of chick skeletal myoblasts is blocked (*8*). These results imply that arginine-specific ADP-ribosylation plays a role in the functions of skeletal muscles.

Little attention has been given to ADP-ribosyltransferase in smooth muscle cells (SMCs). Inhibitors of the transferase block platelet-derived growth factor–induced chemotaxis and the activation of the K^+ channels by 11,12-epoxyeicosatrienoic acid in cultured rat A7r5 SMCs and bovine coronary arterial SMCs, respectively (*9, 10*). Although these results suggest that ADP-ribosyltransferase plays a role in SMCs, no transferase activity was detected in these studies. SMCs undergo change from a differentiated to a dedifferentiated state. For example, SMCs in the media of the arterial wall, normally quiescent and contractile, enter a dedifferentiated state during the early development of atherosclerotic lesions or in primary culture with serum, losing their contractility and acquiring the ability to proliferate and migrate. The ADP-ribosyltransferase activity in SMCs may also change depending on the state of differentiation.

In the present study, we used chicken gizzard smooth muscle to determine whether an arginine-specific ADP-ribosyltransferase activity is present in smooth muscle, and obtained evidence that GPI-anchored arginine-specific ADP-ribosyltransferase locates on the surface of chicken gizzard SMCs. Using an insulin-stimulated culture system

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Abbreviations: SMCs, smooth muscle cells; GPI, glycosylphosphatidylinositol; PI-PLC, phosphatidylinositol-specific phospholipase C; BSA, bovine serum albumin; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HBSS, Hanks' balanced salt solution; PBS, phosphate buffered saline; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; RT-PCR, reverse transcription–polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI3-kinase, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol trisphosphate; TLC, thin layer chromatography; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal protein kinase.

for vascular and visceral SMCs, in which phosphatidylinositol 3-kinase (PI3-kinase) mediates insulin signaling to maintain a differentiated state (*11*), we further investigated the transferase activity in differentiated and dedifferentiated SMCs, and found that the transferase activity decreases dramatically during the process of dedifferentiation, and that PI3-kinase is necessary to maintain the transferase activity in the cells.

MATERIALS AND METHODS

Materials—Chickens were obtained from a local slaughterhouse. Fertilized chicken eggs were incubated at 37°C for 15 days at 60–70% humidity. *Bacillus cereus* PI-PLC, ADP-ribose, HBSS, insulin, poly-L-arginine, and LY294002 were purchased from Sigma. NAD was from Boehringer Mannheim. [Adenylate-^{32}P]NAD (29.6 TBq/mmol) and [^{32}P]orthophosphate (HCl free) were from New England Nuclear.

Methods—All experiments were carried out at least three times and a representative result is shown in the figures and tables.

Fractionation of Gizzard Smooth Muscle and PI-PLC Treatment—Gizzard muscle (1 g) from 6-month-old chickens was homogenized in 10 volumes of medium A containing 0.25 M sucrose, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA, and centrifuged at 600 ×*g* for 10 min. The supernatant was centrifuged at 8,000 ×*g* for 20 min, and then at 100,000 ×*g* for 1 h, and the pellet was suspended in medium A. All procedures were carried out at 4°C. The membrane fraction (5.9 mg/ml, 30.4 nmol/mg/h) was treated with 0.5 unit PI-PLC in 100 μl of medium A at 37°C for 1 h. After centrifugation at 100,000 ×*g* for 1 h, the supernatants were collected and the pellets were resuspended in 100 μl of medium A.

Cell Cultures—SMCs were prepared from 15-day chick embryo gizzards. The isolated cells were seeded onto laminin-coated dishes (FALCON 40405) at a density of 1×10^5 cells/cm^2 and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 0.2% bovine serum albumin (BSA) and 5 μg/ml insulin. The medium was changed daily after day 2. In some cases, the medium was replaced on day 3 with DMEM supplemented with 10% fetal calf serum (FCS) (*12*). On day 7, more than 90% of the cells cultured with FCS were still stained with α-smooth muscle actin antibody. The states of differentiation and dedifferentiation of SMCs were determined based on cell morphology, carbachol-induced contractility, and the expression pattern of caldesmons, as described (*12*).

ADP-Ribosyltransferase Assays—The membrane fraction or the supernatants released from the membrane were incubated with 5 mM NAD and 0.1 M arginine in a reaction mixture containing 50 mM Tris-HCl (pH 9.0), 1 mM EDTA, and 1 mM ADP-ribose in a total volume of 100 μl at 37°C for 3 h. After the reaction was terminated by adding 100 μl of 0.2% SDS, the sample was subjected to capillary electrophoresis (*13*). Zymographic *in situ* gel assay was carried out under non-reducing conditions, as described (*14*) except that the renatured gel was incubated with [^{32}P]NAD and poly-L-arginine at 37°C for 3 h.

ADP-Ribosylation on Cultured SMCs—Cells were washed three times with HBSS and incubated at 37°C for 1 h with 5 μM [^{32}P]NAD (4 Ci/mmol) in 1 ml of HBSS containing 1 mM ADP-ribose, washed three times with cold PBS containing 100 μM unlabeled NAD and 1 mM EDTA, lysed in 100 μl of PBS containing 1% Nonidet P-40, 1 mM ADP-ribose, 1 mM EDTA, and 1 mM PMSF, and centrifuged at 12,000 ×*g* at 4°C for 10 min. The supernatant (140 μg) was subjected to SDS-PAGE in 10% polyacrylamide gels and analyzed using a Fuji BAS 2000. The hydroxylamine sensitivity of the ADP-ribosylarginine linkage was examined, as described (*15*).

Reverse Transcription–Polymerase Chain Reaction (RT-PCR) Analysis—Total RNAs were extracted from precultured or cultured SMCs, and oligo(dT)$_{20}$-primed single-stranded cDNAs were synthesized from 1 μg of total RNAs, using M-MLV reverse transcriptase (TOYOBO). Heat-treated single-strand cDNA mixtures were subjected to PCR using specific primers and rTth DNA polymerase (TOYOBO) under the following conditions: once at 94°C for 2 min and 30 cycles at 94°C for 0.5 min, 52°C for 0.5 min, and 72°C for 2 min for GAPDH. The primer sequences used for PCR were as follows: ACCACAGTCCATGCCATCAC (sense primer) and TCCACCACCCTGTTGCTGTA (antisense primer). PCR conditions and primer sequences for *h*-caldesmon and *l*-caldesmon were as described (*16*). PCR products were separated in 1.2% agarose gels and stained with ethidium bromide.

Phospholipids Analysis—Cells cultured in DMEM containing 0.2% BSA without insulin for 24 h were washed with labeling medium consisting of 10 mM HEPES/NaOH (pH 7.4), 136 mM NaCl, 4.9 mM KCl, 1 mM $CaCl_2$, and 5.5 mM glucose, and incubated at 37°C for 2 h with [^{32}P]orthophosphate (1.85 MBq/ml) in the labeling medium. The cells were then washed twice with the labeling medium and further incubated for 10 min with 1 μM insulin in the labeling medium. Phospholipid extraction and TLC analysis were done as described by Traynor-Kaplan *et al.* (*17*).

Protein Assay—Protein concentration was determined by the dye-binding method of Bradford (*18*) with BSA as a standard.

RESULTS

ADP-Ribosyltransferase Activity in Chicken Gizzard Smooth Muscle—When the whole gizzard homogenate from 6-month-old chickens was incubated with 5 mM NAD in the presence of 100 mM L-arginine and the reaction products were subjected to capillary electrophoresis, peaks corresponding to ADP-ribosylarginine anomers were detected. These peaks were not seen in the absence of L-arginine (data not shown), indicating that an arginine-specific ADP-ribosyltransferase activity is present in gizzard smooth muscle. We next examined the subcellular distribution of the transferase activity in gizzard. Among various fractions tested, the specific activity of the enzyme was highest in the membrane fraction (Table I), indicating that the gizzard smooth muscle transferase is membrane-associated.

Since rabbit skeletal muscle transferase has a GPI-anchor and can be released from the membrane by PI-PLC digestion (*19*), we investigated whether gizzard smooth muscle transferase is also released by PI-PLC. When the membrane fraction from gizzard was incubated with PI-PLC, approximately 80% of the transferase activity originally found in the membrane fraction was detected in the supernatant, while in the absence of PI-PLC only 25% of

the transferase activity was found in the supernatant fraction (Table II). Since these data indicate that smooth muscle transferase is released by PI-PLC treatment, the transferase seems to be GPI-anchored. When the supernatant of the membrane fraction treated with PI-PLC was analyzed by *in situ* zymographic assay, an intense 44 kDa band was observed (Fig. 1, lane 1). The intensity of the band in the pellet fraction was decreased by PI-PLC treatment (data not shown). These data indicate that the apparent molecular mass of the ADP-ribosyltransferase is 44 kDa, as estimated under non-reducing conditions, and that the 44 kDa transferase is released by PI-PLC treatment.

ADP-Ribosyltransferase on the Surface of SMCs—Next, we used a primary culture system for gizzard muscle cells to determine if the arginine-specific ADP-ribosyltransferase is expressed on the surface of gizzard SMCs and modifies cell surface proteins, and whether the transferase activity differs between differentiated and dedifferentiated SMCs.

TABLE I. **Subcellular distribution of arginine-specific ADP-ribosyltransferase in chicken gizzard smooth muscle.** The gizzard muscle was fractionated, and each fraction was incubated with 5 mM NAD and 0.1 M arginine in a reaction mixture containing 50 mM Tris-HCl (pH 9.0), 1 mM EDTA, and 1 mM ADP-ribose at 37°C for 3 h. The amount of product was determined by capillary electrophoresis.

Fractions	Specific activity (nmol/mg/h)
600 ×*g* pellet	8.0
8,000 ×*g* pellet	11.3
100,000 ×*g* pellet	30.1
100,000 ×*g* supernatant	6.6

TABLE II. **PI-PLC–induced release of arginine-specific ADP-ribosyltransferase from the membrane fraction of gizzard smooth muscle.** Membrane fractions were treated with or without PI-PLC. The supernatant or pellet was incubated with 5 mM NAD and 0.1 M arginine in a reaction mixture containing 50 mM Tris-HCl (pH 9.0), 1 mM EDTA, and 1 mM ADP-ribose at 37°C for 3 h, and the amount of product was determined by capillary electrophoresis. The percentage of the activity present in the membrane fractions (13.0 nmol/h) is given in parenthesis.

	ADP-ribosyltransferase activity	
	PI-PLC (–)	PI-PLC (+)
	(nmol/h)	
Supernatant	3.3 (25.4%)	10.5 (80.8%)
Pellet	9.9 (76.2%)	6.2 (47.7%)

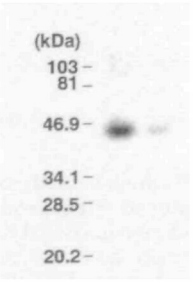

Fig. 1. **Release of a 44 kDa transferase from gizzard smooth muscle membrane by PI-PLC treatment.** The supernatants from the membrane fraction treated with (lane 1) or without (lane 2) PI-PLC were analyzed by *in situ* zymographic assay as described in "MATERIALS AND METHODS." Positions of molecular weight markers are indicated on the left.

Gizzard SMCs from 15-day chick embryos were dispersed by collagenase treatment, and cultured on laminin-coated dishes for 7 days in the presence of insulin. The cells were then incubated with [adenylate-^{32}P]NAD and the lysates were analyzed by SDS-PAGE. Several proteins (135, 110, 68, 64, 56, and 38 kDa) were labeled (Fig. 2A, lane 1). Treatment of the cells with PI-PLC prior to labeling prevented the labeling of these proteins (Fig. 2A, lane 2). In an *in situ* zymographic assay, the 44 kDa band appeared in the PI-PLC–treated supernatant (Fig. 2B, lane 2) concomitant with a reduction in cell labeling, but not so in the non-treated supernatant (Fig. 2B, lane 1). The Trypan blue dye exclusion test confirmed that more than 90% of the cells were viable after PI-PLC treatment. Since the treatment of

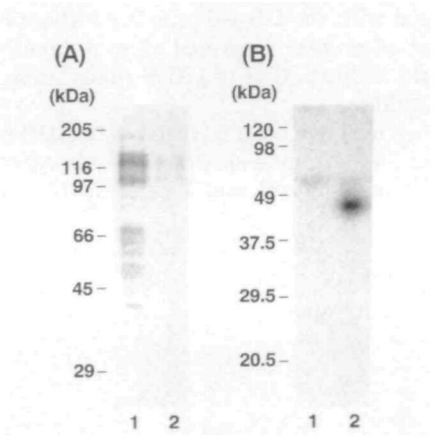

Fig. 2. **ADP-ribosylation of extracellular proteins in differentiated SMCs by GPI-anchored ADP-ribosyltransferase.** (A) Cells on a dish (day 7) were washed with HBSS and treated with (lane 2) or without (lane 1) 1 unit PI-PLC in the same medium (1 ml) at 37°C for 1 h, and then labeled with [^{32}P]NAD. (B) The supernatants of cells treated with (lane 2) or without (lane 1) PI-PLC were condensed by Centricon and analyzed by *in situ* zymographic assay. The renatured gel was incubated with substrates at 37°C for 12 h. Positions of molecular weight markers in A and B are indicated on the left.

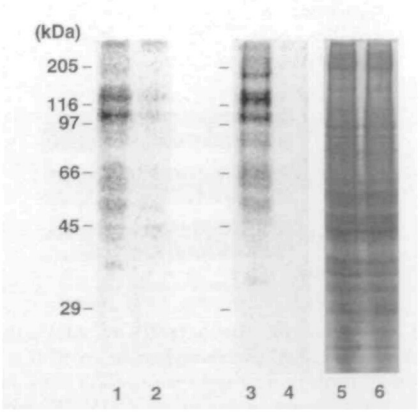

Fig. 3. **Characterization of ADP-ribosylation on differentiated SMCs.** Cells (day 7 after plating) were labeled with [^{32}P]NAD in the absence (lanes 1, 3–6) or presence (lane 2) of 20 mM L-arginine. Acid-insoluble fractions prepared from cells (day 7 after plating) labeled with [^{32}P]NAD were treated with either 1 M hydroxylamine (lanes 4 and 6) or 1 M NaCl (lanes 3 and 5) at 37°C for 2 h, fractionated by SDS-PAGE, stained with Coomassie Brilliant Blue (lanes 5 and 6), and analyzed with a phosphor-imager (lanes 1–4). Positions of molecular weight markers are indicated on the left.

intact cells with PI-PLC releases cell surface GPI-anchored proteins (*20*) and NAD cannot permeate the plasma membrane (*21*), these results indicate that the ADP-ribosyltransferase is, to some extent, present on the surface of SMCs and can modify several cell surface proteins.

To confirm that the modification of these proteins occurs on arginine residues, the effect of L-arginine on the labeling of these proteins and the chemical stability of the radiolabeling were investigated. When SMCs were labeled in the presence of L-arginine, labeling of the proteins was dramatically reduced (Fig. 3, lane 2) as compared with labeling in the absence of L-arginine (Fig. 3, lane 1). The treatment of the labeled proteins with hydroxylamine (Fig. 3, lane 4), which specifically cleaves the ADP-ribosylarginine bond, but not with NaCl (Fig. 3, lane 3) decreased the radioactivity associated with the labeled proteins without decreasing the amount of protein recovered (Fig. 3, lanes 5 and 6). These results indicate that the ADP-ribosylation occurs on arginine residues.

Since integrins have been reported to be ADP-ribosylated by GPI-anchored arginine-specific ADP-ribosyltransferases in mouse skeletal muscle and T cells (*7, 22*), we asked if integrin is also ADP-ribosylated by the transferase in SMCs. After labeling the SMCs with [^{32}P]NAD, β1 integrin, a major integrin subunit associated with several α integrins in chicken SMCs, was immunoprecipitated with anti–β1 integrin antibody. We confirmed that β1 integrin was expressed in the SMCs by immunoblotting with this antibody. However, no radioactivity was detected at the position corresponding to that of the integrin protein (data not shown). Therefore, the major substrates of the transferase in SMCs are neither β1 integrin nor associated α integrins.

Involvement of PI3-Kinase in Maintaining ADP-Ribosyltransferase Activity in Differentiated SMCs—The results described above were obtained using SMCs cultured with insulin. As reported (*12*), when the cells were cultured in the presence of insulin, on day 7, more than 90% of the cells maintained differentiated phenotypes, including carbachol-induced contraction, a spindle-like cell shape (data not shown), and the predominant expression of *h*-caldesmon (Fig. 4C). Under these differentiated conditions, the activity of the transferase (Fig. 4B) or the extent of protein labeling (Fig. 4A) remained unchanged during 7 days of culture. Since the cells were found to dedifferentiate with serum-stimulation (*12*), we investigated the effect of serum in the culture medium on the activity of the transferase and the level of ADP-ribosylation of proteins. Two days after serum addition, the activity of the transferase decreased dramatically (Fig. 4B). There was a corresponding reduction in the labeling of cell surface proteins (Fig. 4A), although we can not rule out the possibility that the substrates for the transferase may be reduced during cell dedifferentiation. We confirmed that at this point the cells became uncontractile and dominantly expressed *l*-caldesmon

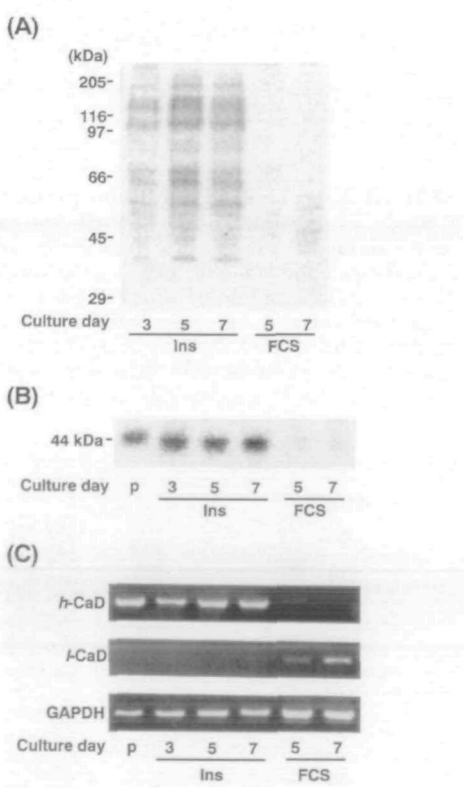

Fig. 4. **Effect of serum on the activity of ADP-ribosyltransferase and the level of ADP-ribosylation in SMCs.** Cells were cultured in DMEM containing 1 μM insulin for 3 days and then further cultured in the same medium (Ins) or in DMEM containing FCS (FCS). (A) On the indicated days, cells were labeled with [^{32}P]NAD as described in "MATERIALS AND METHODS." Molecular weight markers are indicated on the left. (B) Precultured (p) or cultured cells (3×10^6 cells) harvested on the indicated days were treated with 0.5 unit PI-PLC in 100 μl of PBS at 37°C for 1 h. The supernatants/100 μg cell protein were subjected to *in situ* zymography. (C) Total RNAs extracted from cells on the indicated days were subjected to RT-PCR for *h*-caldesmon (*h*-CaD), *l*-caldesmon (*l*-CaD), and GAPDH as described in "MATERIALS AND METHODS."

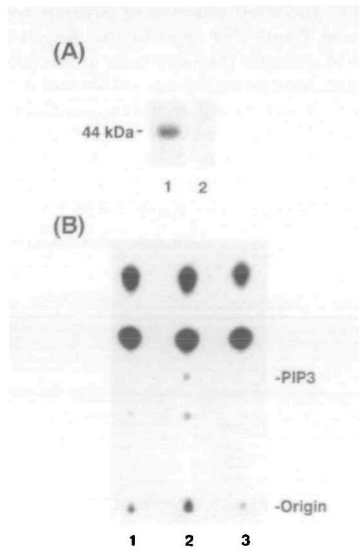

Fig. 5. **Decrease in ADP-ribosyltransferase activity and the inhibition of insulin-induced PIP3 production by LY294002 in SMCs.** (A) Cells were cultured in DMEM containing 1 μM insulin for 24 h and then further cultured in the same medium in the absence (lane 1) or presence of 50 μM LY294002 (lane 2) for 6 days. Cultured cells (3×10^6 cells) were harvested and treated with 0.5 unit PI-PLC in 100 μl of PBS at 37°C for 1 h. The supernatants/100 μg cell protein were subjected to *in situ* zymography. (B) Cells cultured without insulin for 24 h were treated with no addition (lane 1), 1 μM insulin (lane 2), or 1 μM insulin plus 50 μM LY294002 (lane 3) for 10 min. Phospholipids were extracted from the cells and analyzed as described in "MATERIALS AND METHODS."

(Fig. 4C), and the cell shape was fibroblast-like.

Insulin signaling mediated by PI3-kinase plays an essential role in the maintenance of the differentiated phenotype of SMCs (*12*). Therefore, we asked whether PI3-kinase is also required to maintain arginine-specific ADP-ribosyltransferase activity in differentiated SMCs, and we examined the effect of PI3-kinase inhibitor on the activity of the transferase under insulin-stimulated conditions. A PI3-kinase inhibitor, LY294002, suppressed the accumulation of PIP3, a product of PI3-kinase (Fig. 5B), and induced cell dedifferentiation (data not shown) as described previously (*12*). The addition of LY294002 also dramatically reduced the activity of the ADP-ribosyltransferase (Fig. 5A). Similar results were obtained using wortmannin, another structurally unrelated PI3-kinase inhibitor. After treatment with wortmannin for two days, the transferase activity was reduced (data not shown). These results indicate that PI3-kinase is essential for maintaining the activity of arginine-specific ADP-ribosyltransferase in differentiated SMCs.

DISCUSSION

We detected an arginine-specific ADP-ribosyltransferase activity in chicken gizzard smooth muscle. The specific activity of the transferase was highest in the membrane fraction and the transferase was released from the membrane by PI-PLC treatment. *In situ* zymographic assay of ADP-ribosyltransferase revealed a 44 kDa band in the supernatant of the PI-PLC-treated membrane fraction. These results indicate that gizzard smooth muscle contains a GPI-anchored arginine-specific ADP-ribosyltransferase. However, in some cases, a 48 kDa band was observed in the PI-PLC–treated supernatant (data not shown), and PI-PLC resistant activity was detected in the membrane fraction (Table II). Moreover, the transferase activity is present in other fractions (Table I). Thus, other ADP-ribosyltransferases may be present in gizzard smooth muscle. Since the pI value of the transferase was clearly distinct from that of chicken skeletal muscle transferase, which has similar molecular size, these muscular transferases seem not to be identical. We could not determine whether these multiple transferases are derived from the same gene or whether they are post-translationally modified enzymes because the transferase was not identified molecularly.

Whether the GPI-anchored ADP-ribosyltransferase exists on the surface of the cells and whether it modifies cell surface proteins were tested using primary cultured gizzard smooth muscle cells. In this culture, 135-, 110-, 68-, 64-, 56-, and 38-kDa proteins were labeled in the presence of exogenously added [^{32}P]NAD. PI-PLC treatment of the cells before labeling, which released the ADP-ribosyltransferase into culture medium, reduced the labeling. The modification was confirmed to be arginine-specific by the finding that L-arginine inhibited the labeling and was hydroxylamine-sensitive. These results indicate that GPI-anchored arginine-specific ADP-ribosyltransferase is expressed on the surface of differentiated SMCs and that the transferase can ADP-ribosylate the arginine residues of some proteins on the cell surface.

In mouse C2C12 myoblasts and T cells, integrin α7 (*7*) and lymphocyte function-associated molecule-1 (LFA-1) (*22*) were shown to be ADP-ribosylated by the transferase, and integrins may be one of the major substrates for GPI-anchored arginine-specific ADP-ribosyltransferases. In our study using chicken SMCs, the molecular mass of the predominantly ADP-ribosylated proteins (135 kDa) is consistent with that of the integrin subunit expressed on chicken SMCs (*23*). However, we did not obtain data that β1 integrin or associated α integrins are ADP-ribosylated. Other integrins such as β3 integrin or other proteins other than integrins may be modified.

In chicken SMCs, we found that the ADP-ribosyltransferase activity is maintained together with the differentiated phenotype of the cells in the presence of insulin, while the activity decreases dramatically during the process of dedifferentiation stimulated by FCS. Hayashi *et al.* demonstrated that insulin activates PI3-kinase but not mitogen-activated protein kinases (MAPKs), including ERK, JNK, and p38MAPK, in the SMCs, and that a specific PI3-kinase inhibitor, LY294002, suppresses PI3-kinase and induces the dedifferentiation of the SMCs (*12*). We reproduced the effects of LY294002 and found that under the same conditions LY294002 reduces the transferase activity in the cells. Thus, PI3-kinase is necessary to maintain the ADP-ribosyltransferase activity in differentiated SMCs. Since protein kinase B (PKB), a downstream target of PI3-kinase, has been reported to increase the promoter activity of caldesmon, a molecular marker of differentiated SMCs (*11*), the kinase may also increase the transcription of the ADP-ribosyltransferase mRNA. Alternatively, PI3-kinase may post-transcriptionally activate the transferase by activating downstream kinases, including PKB, or may induce the sorting of the transferase to the cell membrane in cells *via* the stimulation of protein trafficking. To clarify the role of PI3-kinase on ADP-ribosyltransferase activity, the molecular identification of the transferase is necessary.

Our data show that the ADP-ribosyltransferase activity is present in SMCs while Saxty *et al.* detected neither the transferase activity nor its message in rat A7r5 SMCs (*9*). The discrepancy regarding the presence of an ADP-ribosyltransferase activity in SMCs may have arisen because the rat SMCs were dedifferentiated since FCS was added to the culture medium. The transferase activity in skeletal muscle increases when myoblasts differentiate into myotubes (*8*), and mature mouse lymphocytes have higher transferase activity than immature cells (*24*). However, the regulatory mechanism governing the transferase activities during differentiation has not been clarified. This is apparently the first report that the insulin signaling pathway mediated through PI3-kinase participates in maintaining the activity of arginine-specific ADP-ribosyltransferase. Since PI3-kinase is also required for skeletal muscle differentiation (*25*), PI3-kinase may play an important role in increasing the transferase activity in both muscles.

As the ADP-ribosyltransferase is expressed predominantly in differentiated SMCs, the transferase may play a physiological role in the cells. Although we investigated the effect of ADP-ribosylation on carbachol-induced contractility, one of the specific functions of differentiated SMCs, the treatment of differentiated SMCs with NAD did not affect carbachol-induced contractility (data not shown). In bovine coronary arterial smooth muscle, inhibitors of ADP-ribosylation block the activation of K^+ channels induced by 11,12-epoxyeicosatrienoic acid (*10*), suggesting that ADP-ribosyltransferase participates in the regulation of K^+ channel activity and in controlling vascular tone in SMCs. Since a

bovine transferase with an unknown amino-acid specificity appears to be present in the cytosolic fraction of SMCs (*10*), the enzyme may differ from the gizzard transferase expressed on the surface of SMCs.

Our evidence shows that a GPI-anchored arginine-specific ADP-ribosyltransferase is expressed on the surface of differentiated chicken gizzard SMCs, and that several cell surface proteins on the cells are modified by this enzyme. Our data also show that the transferase activity decreases dramatically during the process of dedifferentiation and that PI3-kinase is involved in maintaining arginine-specific ADP-ribosyltransferase activity. These results show the presence of an ADP-ribosyltransferase in smooth muscle as well as skeletal muscle, the similarities of these transferases, including GPI-anchorage and the ability to modify some cell surface proteins, and imply the regulatory mechanism of the transferase activity *via* PI3-kinase. We are currently trying to identify the transferase and to examine the effects of ADP-ribosylation on SMC function in differentiated SMCs.

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