Rho-associated kinase (Rho-kinase) from chicken gizzard smooth muscle was purified to apparent homogeneity (160 kDa on SDS-polyacrylamide gel electrophoresis) and identified as the ROKα isoform. Several substrates were phosphorylated. Rates with myosin phosphatase target subunit 1 (MYPT1), myosin, and the 20-kDa myosin light chain were higher than other substrates. Thiophosphorylation of MYPT1 inhibited myosin phosphatase activity. Phosphorylation of myosin at serine 19 increased actin-activated Mg\(^{2+}\)-ATPase activity, i.e. similar to myosin light chain kinase. Myosin phosphorylation was increased at higher ionic strengths, possibly by formation of 6 S myosin. Phosphorylation of the isolated light chain and myosin phosphatase was decreased by increasing ionic strength. Rho-kinase was stimulated 1.5–2-fold by guanosine 5′-O-3-(thio)triphosphate-RhoA, whereas limited tryptic hydrolysis caused a 5–6-fold activation, independent of RhoA. Several kinase inhibitors were screened and most effective were Y-27632, staurosporine, and H-89. Several lipids caused slight activation of Rho-kinase, but arachidonic acid (30–50 μM) induced a 5–6-fold activation, independent of RhoA. These results suggest that Rho-kinase of smooth muscle may be involved in the contractile process via phosphorylation of MYPT1 and myosin. Activation by arachidonic acid presents a possible regulatory mechanism for Rho-kinase.

Contraction of smooth muscle is regulated primarily by the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) and phosphorylation of the 20-kDa myosin light chain (MLC20) by Ca\(^{2+}\)-calmodulin-dependent myosin light chain kinase (MLCK) (1–4). However, the relationship between [Ca\(^{2+}\)], and myosin phosphorylation is not fixed and under certain conditions may shift. Increased phosphorylation at submaximal Ca\(^{2+}\) levels, referred to as increased Ca\(^{2+}\) sensitivity, frequently occurs following agonist stimulation (4). With respect to the balance of kinase and phosphatase activities, this effect could reflect either increased activity of the Ca\(^{2+}\)-calmodulin-MLCK complex or, inhibition of myosin phosphatase (MP). The latter was found to be the case (5, 6) and it was suggested that the inhibition of MP involved a G-protein-linked pathway (4). In addition, several studies showed that the small GTPase Rho, specifically RhoA (7), was important in smooth muscle function and the increased Ca\(^{2+}\) sensitivity following agonist stimulation (for reviews, see Refs. 4 and 8).

Recently several target proteins of Rho have been identified, including protein kinase N (9, 10), Rho-associated kinase (Rho-kinase) (11–13), rhophilin (10), rhotekin (14), citron (15), p140mDia (16), and MP target subunit 1, termed MYPT1 (17). Phosphorylation of MYPT1 by Rho-kinase inhibited MP and this was suggested as a mechanism to increase the Ca\(^{2+}\) sensitivity in smooth muscle (17). In addition, it was found that bovine brain Rho-kinase could directly phosphorylate myosin in vitro (18) and thus the change in Ca\(^{2+}\) sensitivity could reflect the two phosphorylation mechanisms, i.e. MYPT1 and myosin. Indeed, introduction of the constitutively active recombinant fragment of bovine Rho-kinase into Triton X-100-permeabilized smooth muscle provoked a Ca\(^{2+}\)-independent contraction via MLCK phosphorylation (19). Also, a specific inhibitor of Rho-kinase, Y-27632, was reported to inhibit the agonist-induced Ca\(^{2+}\)-sensitization of smooth muscle contraction (20). Rho-kinase has been reported to be involved in other cellular functions including the formation of stress fibers and focal adhesions (21, 22) and cardiac hypertrophic responses (23).

Rho-kinase has been classified into two isoforms: ROKα and ROKβ (12, 21), also referred to as ROCK-II and ROCK-I (24), respectively. The isoforms are relatively large proteins (150–160 kDa) consisting of an amino-terminal kinase domain, a central coiled-coil domain that includes the Rho-binding domain, and a carboxyl-terminal putative pleckstrin homology domain separated by the insertion of a cysteine-rich zinc finger domain (11–13, 21, 24). Purification protocols have been described for the isolation of Rho-kinases from rat brain (11), bovine brain (12), and human platelets (13). However, it is surprising that despite the numerous reports supporting a role for RhoA in smooth muscle there are no data available on the smooth muscle Rho-kinase. In the present study, we have purified the smooth muscle Rho-kinase from chicken gizzard. It is suggested that the gizzard Rho-kinase is the ROKα isoform. The biochemical properties of the smooth muscle Rho-kinase
were combined and polyethylene glycol 6000 added to 6% (v/v). After 30 min and dissolved in 200 ml of buffer B (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 mM EGTA, 0.1 mM dithiothreitol, 1 mM benzamidine, 0.2 μM (p-aminophenyl) methanesulfonfonyl fluoride, and 5% glycerol) for 2 h. After brief centrifugation (10,000 × g for 5 min) the sample was loaded onto a phosphocellulose column (1.6 × 10 cm) equilibrated in buffer C. The column was washed with 5柱 volumes of buffer C plus 0.15 M NaCl and Rho-kinase was eluted (at 0.45 × NaCl) using 0.15 M NaCl with 0.1 M diithiothreitol and a linear gradient of NaCl (0.15–0.7 M) in buffer C. Fractions were pooled and dialyzed overnight against buffer D (10 mM PIPES-NaOH, pH 6.0, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM benzamidine, 0.2 μM (p-aminophenyl) methanesulfonfonyl fluoride, and 5% glycerol). The dialyzed sample was applied to a 1-ml glutathione-Sepharose 4B column immobilized with 5 mg of the “active” form of RhoA, i.e., GTPγS-RhoA (12). The column was washed with 10 volumes of buffer D containing 0.5 M NaCl. Rho-kinase was eluted in two steps: initially by elution at high pH (30 mM Tris-HCl, pH 8.3, 0.1 mM dithiothreitol) and subsequently by elution with gluthathione (30 mM Tris-HCl, pH 7.5, 10 mM glutathione, and 0.1 mM dithiothreitol).

**Protein Kinase Assay**—Unless otherwise indicated, Rho-kinase activity was assayed in a reaction mixture (final volume of 50 μl) containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM dithiothreitol, 5 mM MgCl₂, 1 mM EDTA, 1 μM microcystin-LR, enzyme, and substrate as indicated. MLCK activity was assayed in a reaction mixture (final volume of 50 μl) containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM dithiothreitol, 1 mM MgCl₂, 1 μM microcystin-LR, 2 μM calmodulin, and 2 μM cyclic AMP. The reactions were initiated by addition of [γ-32P]ATP to a final concentration of 100 μM. After incubation at 30 °C for 5 min (enzyme concentration was adjusted to ensure linear kinetics at this time) 40-μl aliquots were removed and added to phosphocellulose paper. The paper was washed immediately for 10 min with 75 mM phosphoric acid (4 times), dried, and 32P incorporation determined by Cerenkov counting. Alternatively, reactions were terminated by addition of Laemmli sample buffer and boiled for 3 min. Samples were subjected to SDS-PAGE (7.5–20%), followed by autoradiography or 32P determination in excised gel slices.

**MP Assay**—Phosphatase assays were carried out at 30 °C using 32P-labeled MLCK (final concentration 5 μM) as substrate. Assay conditions were: 30 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.2 mg/ml bovine serum albumin, and 0.5 mM CaCl₂ (26).

**Phosphorylation of Myosin**—Phosphorylation of MP holenzyme by Rho-kinase was carried out at 30 °C for different times in 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1 mM dithiothreitol, 5 mM MgCl₂, 1 mM EDTA, 30 μg/ml MP, 2 μg/ml Rho-kinase, 2 μM GTPγS-GST-RhoA, and 100 μM [32P]ATP in the presence or absence of 1 μM microcystin-LR in a final volume of 50 μl. The reactions were initiated by addition of [32P]ATP and terminated by addition of Laemmli sample buffer and immediately boiled for 3 min. These samples were subjected to SDS-PAGE (7.5–20%), followed by autoradiography or 32P determination in excised gel slices, positions corresponding to MYPT1, PP1cδ, or M20. To determine the effects of phosphorylation on MP activity, phosphorylation of MP was carried out under the similar conditions without microcystin-LR using ATP instead of ATP. At each time point a sample was removed, diluted 100-fold with a buffer containing 30 mM Tris-HCl, pH 7.5, 0.2 mg/ml bovine serum albumin, and 0.1 mM EDTA and assayed for phosphatase assay. Phosphorylation of the recombiant fragments of MYPT1 (30 μg/ml r-MYPT167–1004, 30 μg/ml rMYPT11–1004, and 20 μg/ml GST) was carried out in the presence or absence of 2 μg GTPγS-GST-RhoA and in the absence of microcystin-LR under the above conditions.

To determine the stoichiometry of phosphorylation of myosin, myosin (1 mg/ml) was incubated with 250 μM [γ-32P]ATP in 20 mM Tris-HCl, pH 7.5, 300 mM KCl, 0.1 mM dithiothreitol, 1 mM MgCl₂, 2 μg/ml Rho kinase, and 2 μM GTPγS-GST-RhoA, final volume 50 μl. After incubation for different times, reactions were terminated by addition of trichloroacetic acid and sodium pyrophosphate to 5 and 1%, respectively. The precipitated protein was collected and washed with the trichloroacetic acid/sodium pyrophosphate solution, using plastic funnels (Seapac columns, Seikagaku Kogyo, Tokyo, Japan) fitted with a fiberglass disc and cotton plug (27). 32P incorporation was estimated by Cerenkov counting. Assays of actin-activated Mg²⁺-ATPase activity of myosin were as described previously (32).

**Peptide Sequence Tagging with Muscle Rho-kinase**—Purified Rho-kinase was subjected to 6% SDS-PAGE and transferred to a Polytron® membrane (Applied Biosystems). The band corresponding to Rho-kinase was digested by lysyl endopeptidase. The resulting peptides were separated by high performance liquid chromatography and subjected to a gas-phase sequencer (Shimadzu PSSQ-1, Tokyo, Japan) as described previously (33).

**Other Procedures**—Lipids were routinely prepared as chloroform,
ethanol, or dimethyl sulfoxide stocks, and vesicles were prepared as described earlier (25). For dimethyl sulfoxide stocks, lipids were directly diluted into the same buffer. SDS-PAGE was carried out with the discontinuous buffer system of Laemmli (34). Western blotting analyses were carried out as described previously (25, 35). The polyclonal antibody to the NH2-terminal region of MYPT1 (17) and to the coiled-coil region of bovine Rho-kinase (36) were prepared as described earlier. The anti-Rho-kinase antibody recognized both ROKα and ROKβ isoforms. Protein concentrations were determined with either the BCA (Pierce) or Bradford (Bio-Rad) procedures, using bovine serum albumin as a standard.

RESULTS

Purification of Rho-kinase from Chicken Gizzard—The distribution of Rho-kinase in various fractions from gizzard was investigated using the bovine Rho-kinase antibody. Most of the kinase was present in the cytosolic fraction (75.3 ± 3.3%; mean ± S.E., n = 3) compared with the membrane (20.4 ± 2.9%) and nuclear fractions (4.3 ± 1.0%).

Gizzard cytosol was used as source for purification of Rho-kinase. The purification procedure was monitored by Western blots and also by assay with GTPγS-GST-RhoA and rG-MYPT1667–1004 as substrate. An efficient (high yield) method was developed for purification of Rho-kinase as outlined in Table I. The yield of Rho-kinase from 200 g of chicken gizzard was approximately 33 μg and this was purified over 20,000-fold to apparent homogeneity.

The initial step was precipitation with polyethylene glycol 6000 and this recovered about 80% of the cytosolic Rho-kinase. The elution profiles of the subsequent chromatography procedures are shown in Fig. 1. A. Q-Sepharose FF chromatography. B. phosphocellulose chromatography. C. GTPγS-GST-RhoA affinity chromatography on glutathione-Sepharose 4B. Aliquots of fractions from each step were assayed in the presence of 2 μM GTPγS-GST-RhoA (●) or GDP-GST-RhoA (○) using 0.1 mg/ml rG-MYPT1667–1004 as substrate under standard assay conditions as described under “Experimental Procedures.” D, eluate by pH 8.3 (a) or 10 mM glutathione (b) from GTPγS-GST-RhoA affinity column. Lanes 1 and 3, Coomassie Blue stains; lanes 2 and 4, Western blots using anti-Rho-kinase antibody; lane 5, Western blot using anti-MYPT1 antibody.

| Fraction          | Volume (ml) | Protein (mg) | Total activity (nmol/min) | Specific activity (nmol/min/mg) | Purification Fold | Yield (%) |
|-------------------|-------------|--------------|---------------------------|---------------------------------|------------------|-----------|
| Homogenate        | 1826 ± 36   | 10502 ± 658  | 79.4 ± 11.7               | 0.0072 ± 0.0017                 | 1                 | 100       |
| 6% Polyethylene glycol | 183 ± 16     | 1965 ± 156   | 66 ± 40                   | 0.0324 ± 0.0048                 | 4.5 ± 0.2         | 83 ± 4    |
| Q-Sepharose FF    | 122 ± 10    | 223 ± 9      | 42 ± 28                   | 0.23 ± 0.03                     | 30.8 ± 1.3        | 64 ± 5    |
| Phosphocellulose  | 50.3 ± 1.5  | 18.6 ± 3.1    | 27 ± 21                   | 1.38 ± 0.04                     | 208 ± 24          | 40 ± 12   |
| RhoA affinity     | 5.0 ± 0.5   | 0.033 ± 0.004 | 4.1 ± 2.9                | 140.5 ± 8.2                     | 20508 ± 186       | 5.6 ± 0.4 |

Fig. 1. Purification of smooth muscle Rho-kinase. A, Q-Sepharose Fast Flow chromatography. B, phosphocellulose chromatography. C, GTPγS-GST-RhoA affinity chromatography on glutathione-Sepharose 4B. Aliquots of fractions from each step were assayed in the presence of 2 μM GTPγS-GST-RhoA (●) or GDP-GST-RhoA (○) using 0.1 mg/ml rG-MYPT1667–1004 as substrate under standard assay conditions as described under “Experimental Procedures.” D, eluate by pH 8.3 (a) or 10 mM glutathione (b) from GTPγS-GST-RhoA affinity column. Lanes 1 and 3, Coomassie Blue stains; lanes 2 and 4, Western blots using anti-Rho-kinase antibody; lane 5, Western blot using anti-MYPT1 antibody.
Smooth Muscle Rho-kinase

was obtained using baculovirus-expressed RhoA, which was determined and compared with matching sequences from rat ROKα (Refs. 11 and 21; also referred to as ROCK-II, see Ref. 24). The activity of smooth muscle Rho-kinase toward the small noncatalytic subunit of MP, M20 (data not shown).

The co-purification of MP and Rho-kinase from the Rho affinity column suggested that both interacted with the active form of RhoA, i.e. GTPγS-GST-RhoA. To confirm this, a precipitation assay using GTPγS-GST-RhoA-coupled to glutathione-Sepharose 4B and an overlay assay using [35S]GTPγS-GST-RhoA were carried out. These assays showed that Rho-kinase and MYPT1 interacted specifically with GTPγS-GST-RhoA, but interactions were not detected with either GDP-GST-RhoA nor GTPγS-GST-RhoA (data not shown).

To identify the isoform of the purified smooth muscle Rho-kinase, the p160 was subjected to proteolysis and sequence analysis of derived peptides. The sequences of 3 peptides were determined and compared with matching sequences from rat ROKα (Refs. 11 and 21; also referred to as ROCK-II, see Ref. 24) and rat ROKβ (also referred to as ROCK-I). As shown in Fig. 2, the p160 more closely matched ROKα (28 of 30 residues identical) than ROKβ (21 of 30 residues identical). Thus the Rho-kinase isolated from gizzard was the ROKα isoform.

**Enzymatic Properties of Smooth Muscle Rho-kinase**—Smooth muscle Rho-kinase phosphorylates a number of proteins, as do several protein kinases. As shown in Table II the smooth muscle Rho-kinase phosphorylated MYPT1, myosin, MLCK, myofilament basic protein, protamine, histone IIIs, and histone VIIIs. Among these MYPT1, myosin and MLCK20 appeared to be better substrates. MLCK, caldesmon, and tropomyosin were not phosphorylated by smooth muscle Rho-kinase under the same conditions. The activity of smooth muscle Rho-kinase toward each substrate was stimulated by GTPγS-GST-RhoA 1.5–2-fold (Table II). A similar result (1.9±0.34-fold activation, n = 4) was obtained using baculovirus-expressed RhoA, which was genanyclegenylated at the COOH terminus. K_m, V_max, and k_cat values of smooth muscle Rho-kinase for MLCK20 and GMP-MYPT1677–1004 are shown in Table III. These were estimated at 100 μM ATP. For Rho-kinase and MLCK20 the K_m and V_max values obtained at 200 μM ATP were similar (Table III). In general, GTPγS-GST-RhoA causes a slight decrease in K_m (reduced by about half) and an increase in V_max and k_cat (1.4–1.6-fold). The K_m value of smooth muscle Rho-kinase for ATP was 30.8±6.2 μM (mean ± S.E., n = 4). This value is considerably higher than that reported for the recombinant human p160ROCK, i.e. 0.1 μM (20).

To examine the effects of pH on Rho-kinase activity, assays were carried out over a range of pH of 4 to 9.3. As shown in Fig. 3A, the basal activity of Rho-kinase (in the absence of GTPγS-GST-RhoA) was maximum at pH 6.0. An interesting point is that at pH 6.0 the activity of Rho-kinase was independent of GTPγS-GST-RhoA, whereas at pH 7.5 the dependence on Rho-kinase was observed (Inset of Fig. 3A). The activity of Rho-kinase at pH 6.0, either in the presence or absence of GTPγS-GST-RhoA, was similar to that shown in the presence of GTPγS-GST-RhoA at pH 7.5. The effects of ionic strength on Rho-kinase activity with myosin, MLCK20, and the MP holoenzyme as substrates are shown in Fig. 3B. With intact myosin (and 1 mM MgCl_2) activity was increased with increasing ionic strength and reached a maximum at 0.3 M KCl. A sharp decline in activity occurred after 0.5 M KCl. With MLCK20 or MP as substrate, increasing ionic strength caused a gradual decrease in Rho-kinase activity.

Next the effect of various protein kinase inhibitors on gizzard Rho-kinase was investigated. K values for smooth muscle Rho-kinase were estimated by Dixon plots and are summarized in Table IV. Staurosporine (a wide-range inhibitor of protein kinases (37)) was the most potent inhibitor and its K value was about 0.02 μM. Y-27632, an inhibitor of p160ROCK (20) and H-89 (an inhibitor of protein kinase A (38)), also strongly inhibited smooth muscle Rho-kinase. Other inhibitors were less effective and these included: H-7 and A3 (38); W-7 (a calmodulin antagonist (39)); and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (an inhibitor of casein kinase II (40)). H-89 (38) even at 100 μM showed only slight inhibition. The smooth muscle Rho-kinase was not inhibited (data not shown) by millimolar concentrations of chelerythrine (an inhibitor of protein kinase C (41)) and wortmannin (an inhibitor of phosphatidylinositol 3-kinase and MLCK (42)).

**Effects of Phosphorylation of MP and Myosin**—The time course and stoichiometry of phosphorylation for MYPT1 of MP by smooth muscle Rho-kinase is shown in Fig. 4A. MYPT1 was phosphorylated up to 1.8 and 1.3 mol of P/mol of MYPT1 in the

### Table II

| Substrate (2 μM) | Activity |
|-----------------|----------|
|                  | GTPγS·GST-RhoA (−) | GTPγS·GST-RhoA (+) |
| MYP1            | 147 ± 24 | 203 ± 42 |
| MLCK20          | 112 ± 17 | 167 ± 36 |
| Myosin*         | 138 ± 14 | 276 ± 63 |
| Caldesmon       | Negligible | Negligible |
| MLCK            | Negligible | Negligible |
| Tropomyosin     | Negligible | Negligible |

* Myosin was phosphorylated at 1 mM MgCl_2 and 0.3 mM KCl. Values are mean ± S.E. (n = 4).

### Table III

**Comparison of the kinetics of Rho-kinase and MLCK**

| Enzyme/substrate | K_m substrate | V_max | k_cat a |
|------------------|---------------|-------|---------|
| Rho-kinase/MLCK20 | 4.51 ± 0.12 | 480 ± 51 | 76.8 ± 8.2 |
| + GTPγS·GST-RhoA | (5.96) b | (530) b | |
| + Arachidonic acid | 24.8 ± 1.1 | 3246 ± 177 | 519.5 ± 20.6 |
| Rho-kinase/rG-MYPT1677–1004 | 0.18 ± 0.03 | 545 ± 7 | 87.3 ± 1.8 |
| + GTPγS·GST-RhoA | 0.10 ± 0.01 | 883 ± 42 | 141.4 ± 6.2 |
| + Arachidonic acid | 0.18 ± 0.02 | 6564 ± 374 | 1050.3 ± 59.9 |
| MLCKM20 | 34.5 ± 2.8 | 2148 ± 53 | 230.9 ± 5.7 |

* The molecular sizes used were 160 kDa for Rho-kinase and 108 kDa for MLCK.

### Table I

**Alignment of the smooth muscle Rho-kinase-derived peptide sequences with ROKα and ROKβ.** Three peptides sequences derived from smooth muscle Rho-kinase revealed a highly significant homology with rat ROKα. Residues are shown white on black when at least two out of three are identical.
presence and absence of 1 μM microcystin-LR, respectively. M20 was not an effective substrate and PP1c was not phosphorylated under the same conditions (Fig. 4A). Thio phosphorylation of MYPT1 of MP by Rho-kinase decreased the phosphatase activity toward 32P-MLC20 (Fig. 4B). Using the recombinant fragments of chicken MYPT1 (M133 isoform (31)) as substrates, it was shown that the smooth muscle Rho-kinase phosphorylated the COOH-terminal part of MYPT1 (residues 667–1004) and the NH2-terminal fragment was not phosphorylated (Fig. 4C). These data indicate that there are at least 2 phosphorylation sites for Rho-kinase in the COOH-terminal part (Ser667 to Ile1004) of MYPT1.

The time course and stoichiometry of myosin phosphorylation by Rho-kinase are shown in Fig. 5A. In the presence of GTPγS-GST-RhoA, myosin was maximally phosphorylated by Rho-kinase to 1.8 mol of Pi/mol of myosin at 0.3 M KCl. A semilogarithmic plot of the time course indicated that the phosphorylation of myosin by Rho-kinase followed two first-order processes. Autoradiograms showed that only MLC20 was phosphorylated (data not shown), and phosphoamino acid analysis showed that the major site of phosphorylation was phosphoserine (data not shown). Two-dimensional phosphopeptide mapping analysis indicated only one major phosphopeptide and the phosphopeptide map was identical to that observed on phosphorylation of myosin (or MLC20) with MLCK (data not shown). These results suggested that smooth muscle Rho-kinase phosphorylates MLC20 at Ser19, i.e. the major site for phosphorylation of myosin by Rho-kinase for 30 min in the presence (lanes even number) or absence (lanes uneven number) of microcystin-LR and then phosphatase assays were carried out as described under “Experimental Procedures.” Myosin phosphatase activity, prior to preincubation with ATPγS (0 min), was expressed as 100%. C, phosphorylation of recombinant fragments of MYPT1. GST (lanes 1, 2, 7, and 8), rMYPT1(667–1004) (lanes 3, 4, 9, and 10), and rG-MYPT1(667–1004) (lanes 5, 6, 11, and 12) were phosphorylated by Rho-kinase for 30 min in the presence (lanes even number) or absence of GTPγS-GST-RhoA (lanes uneven number) as described under “Experimental Procedures.” The phosphorylated samples were then subjected to 7.5–20% SDS-PAGE and visualized by Coomassie Blue staining (lanes 1–6) and autoradiography (lanes 7–12).

MLCK (1–3). The effect of phosphorylation of myosin by Rho-kinase on actin-activated ATPase activity was determined and compared with that by MLCK. As shown in Fig. 5B, phosphorylation of myosin by Rho-kinase increased the actin-activated ATPase activity of myosin, but not by MLCK. These results suggest that the phosphorylation of myosin by Rho-kinase in the presence of ATPγS-GST-RhoA by Rho-kinase with (●, ▲) or without (○) 1 μM microcystin-LR, and 32P incorporation in the excised gel slices corresponding to the position of MYPT1 (●, ○), M20 (▲), and PP1c68 (■) were determined as described under “Experimental Procedures.” B, myosin phosphatase holoenzyme were preincubated with ATPγS for the indicated time without microcystin-LR and then phosphatase assays were carried out as described under “Experimental Procedures.” Myosin phosphatase activity, prior to preincubation with ATPγS (0 min), was expressed as 100%. C, phosphorylation of recombinant fragments of MYPT1. GST (lanes 1, 2, 7, and 8), rMYPT1(667–1004) (lanes 3, 4, 9, and 10), and rG-MYPT1(667–1004) (lanes 5, 6, 11, and 12) were phosphorylated by Rho-kinase for 30 min in the presence (lanes even number) or absence of GTPγS-GST-RhoA (lanes uneven number) as described under “Experimental Procedures.” The phosphorylated samples were then subjected to 7.5–20% SDS-PAGE and visualized by Coomassie Blue staining (lanes 1–6) and autoradiography (lanes 7–12).

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myosin on actin-activated ATPase activity. Myosin (1 mg/ml) was phosphorylated by Rho-kinase (E) by Rho-kinase and MLCK were 1.8 and 1.9 mol of Pi/mol of myosin, respectively. The actin-activated ATPase activity was measured as described previously (32).

Regulation of Smooth Muscle Rho-kinase—Incubation of the purified enzyme with [γ-32P]ATP under the standard assay conditions resulted in autophosphorylation of Rho-kinase. About 1.6 and 1.2 mol of phosphate/mol of Rho-kinase were incorporated in the presence and absence of GTPγS-GST-RhoA, respectively (data not shown). The activity of Rho-kinase and the activation by GTPγS-GST-RhoA were not affected by autophosphorylation (data not shown).

Digestion of Rho-kinase with trypsin (1:500, w/w, trypsin:Rho-kinase) resulted in a marked increase of activity. As shown in Fig. 6, the dependence of Rho-kinase activity on GTPγS-GST-RhoA initially was lost (after approximately 7 min digestion) and continued proteolysis caused an increase in the GTPγS-GST-RhoA-independent activity. The increase in constitutive activity, i.e. in the absence of GTPγS-GST-RhoA, reached a maximum that was 5–6-fold higher than the native enzyme. Further proteolysis decreased the Rho-kinase activity (Fig. 6). SDS-PAGE analyses of the tryptic digests showed that the 160-kDa band was degraded initially into a 125-kDa fragment, and further cleavage generated 3 major products of 112, 88, and 77 kDa (inset of Fig. 6). Maximum activation of Rho-kinase activity was closely correlated with the appearance of the 112- and 88-kDa fragments, indicating that these fragments contained the catalytic domain.

The pleckstrin homology domain has been reported to be involved in lipid binding (43, 44). This prompted an investigation of the effects of lipids on the activity of Rho-kinase (Figs. 7A and 6B). Arachidonic acid caused a marked stimulation of Rho-kinase activity and some acidic phospholipids induced a smaller activation (Fig. 7A) with MLCK as substrate. The concentration dependence for arachidonic acid and the acidic phospholipids are shown in Fig. 7B. An activation of about 6-fold was achieved by 30–50 μM arachidonic acid and over the same concentration range phosphatidylinositol induced about a 2.5-fold activation. The level of Rho-kinase activity achieved by arachidonic acid was about the same as that induced by tryptic hydrolysis. The activation of Rho-kinase was observed with arachidonic acids obtained from several commercial sources. Activation of Rho-kinase by arachidonic acid was observed also using the MYPT1 substrate, rG-MYPT1667–1004, as shown in Table III. kcat was increased about 12-fold, compared with basal activity, by 50 μM arachidonic acid. Phosphatidylycerine, phosphatidic acid, lysophosphatidylether, lysophosphatidic acid, and phosphatidylcholine, 4,5-bisphosphate also stimulated Rho-kinase activity (2–3-fold) but at higher lipid concentrations (Fig. 7B). Sphingosine and sphingosine 1-phosphate slightly stimulated Rho-kinase activity (Fig. 7A). Other lipids did not affect the activity of Rho-kinase (Fig. 7A). The stimulation of Rho-kinase activity by arachidonic acid, or acidic phospholipids, was independent of GTPγS-GST-RhoA and the maximum activation by arachidonic acid was not affected by the presence of GTPγS-GST-RhoA. Thus activation of Rho-kinase by lipids offers an alternative potential regulatory mechanism that is independent of the small G proteins.
activation of Rho-kinase activity and this reflects a decrease in $K_m$ and an increase in $V_{max}$ (or $k_{cat}$). However, this is a relatively modest degree of activation, 1.5–2-fold, compared with regulatory influences with other protein kinases. The reported effects of GTP-RhoA are variable, ranging from zero activation (11), 2-fold activation (13) to about 15-fold activation using a fragment of MYPT1 (12). Several factors may influence basal activity (in the absence of GTP-RhoA) and activation by GTP-RhoA. These include: the substrate used (12), preactivation during Rho-affinity chromatography (11), and other modifications incurred during isolation, and autophosphorylation. A precedent to support the latter is the autophosphorylation of p21-activated serine/threonine kinase that reduces binding of Cdc42 or Rac and activates kinase activity (45). Autophosphorylation of Rho-kinase has been reported (see Refs. 11–13, and above data) but from our results this did not affect kinase activity or the binding of GTP-S bound RhoA. It cannot be eliminated that autophosphorylation of the gizzard Rho-kinase occurred before its isolation. It might also be argued that translocation of Rho-kinase from the cytosol to the membrane-bound GTP-RhoA could result in a more effective regulation by RhoA. The assembly of a multiprotein complex (46, 47) at the membrane may result in altered protein conformations or the contribution to the regulatory mechanism of membrane components.

The above discussion raises the possibility that there could be other regulatory mechanisms for Rho-kinase, either independent of or complementary to RhoA. Limited digestion of Rho-kinase by trypsin causes 5–6-fold activation of RhoA-independent kinase activity. It was suggested (21) that the pleckstrin homology/cysteine-rich zinc finger domain contained an autoinhibitory sequence(s) and thus activation via tryptic hydrolysis could be explained by loss of this inhibitory sequence and the generation of a constitutively active NH$_2$-terminal kinase fragment. The activity of this fragment is considerably higher than that achieved by GTP-S-GST-RhoA and raises the question of whether another modulator/regulator might be involved in realizing full activation of Rho-kinase. This, together with the possibility that membrane components could be involved, prompted our investigation of the effects of various lipids on Rho-kinase activity. An interesting finding was that Rho-kinase was activated 5–6-fold by arachidonic acid and to a lesser extent by certain acidic phospholipids. The activation was independent of RhoA. The activation of Rho-kinase by arachidonic acid is intriguing since the first mechanism proposed for increased Ca$^{2+}$ sensitivity in smooth muscle involved arachidonic acid. It was shown in permeabilized muscle fibers that arachidonic acid increased force and MLC20 phosphorylation. A precedent to support the latter is the autophosphorylation of the gizzard Rho-kinase and an increase in $V_{max}$ (or $k_{cat}$).

**DISCUSSION**

The purification and partial characterization of Rho-kinase from chicken gizzard is described above and to our knowledge is the first report of the isolation of Rho-kinase from smooth muscle. The isolation procedure is relatively simple and provides Rho-kinase close to homogeneity and in reasonable yields. Most of the Rho-kinase was present in the cytosolic fraction and previous preparations of Rho-kinase have used both the cytosol (e.g. from platelets (13)) and the membrane fraction (e.g. from brain (12)). The enzyme isolated from gizzard was the ROKa isoform. The 2 isoforms of Rho-kinase have some differences in tissue distribution. ROKb (ROCK-I) was detected in many tissues, although at higher levels in liver, lung, and testis. In contrast, ROKa (ROCK-II) was enriched in brain and skeletal muscle (11–13, 21, 24).

The properties of the gizzard Rho-kinase are similar to those reported earlier. The mass (as judged from SDS-PAGE) is about 160 kDa and it binds to RhoA in the GTP, or GTP-S, bound form. Binding of the active form of RhoA results in
any role for arachidonic acid would probably be downstream of Rho activation.

Potential substrates for the bovine ROK α isoform include: MYPT1 (17), MLC20 (18), α-adducin (36), and ERM family (ezrin, radixin, and moesin (51)). With respect to the Ca^{2+} sensitivity of smooth muscle the focus is on MYPT1 and MLC20 and the gizzard Rho-kinase showed high activity toward both. With the rG-MYPT1 fragment the $k_c$ for gizzard Rho-kinase was considerably lower than the estimated concentration of MYPT1 in smooth muscle, about 1 $\mu$M (8) and thus MYPT1 is a reasonable in vivo target for Rho-kinase. As shown above, thio phosphorylation of MYPT1 by Rho-kinase inhibited the MP holoenzyme, confirming earlier observations (17). The sites of phosphorylation on MYPT1 have not been established and this is under investigation. Thus consistent with earlier suggestions (12, 17) it is proposed that a major target for Rho-kinase is the MYPT1 subunit. In a previous report it was found that MYPT1 was phosphorylated by an endogenous kinase in the MP holoenzyme preparation (52). Phosphorylation at Thr^{657}/Thr^{695} of the M130/M133 isoforms, respectively, inhibited phosphatase activity. The endogenous kinase was inhibited by chelerythrine (IC_{50} 5 $\mu$M) but not by H-7. In contrast, the smooth muscle Rho-kinase was inhibited by H-7 ($K_i$ 0.45 $\mu$M) but not by millimolar concentration of chelerythrine. Clearly, Rho-kinase and the endogenous kinase are not identical. It is apparent, therefore that more than one kinase can phosphorylate MYPT1 and cause inhibition of MP.

The second substrate for Rho-kinase that potentially is important in the contractile process of smooth muscle is myosin. It is shown above that Rho-kinase phosphorylates MLC20 and intact myosin and that the time course of phosphorylation of myosin followed two rates, implying sequential phosphorylation of the two heads of myosin. This is similar to the situation with myosin and MLCK (1, 53). The increase in the myosin phosphorylation rate on increasing ionic strength may reflect a change in myosin conformation, namely the 10 S to 6 S transition that occurs between 0.2 and 0.3 M KCl (54). Previously it was reported that brain Rho-kinase phosphorylated myosin (18) and our data confirm this observation with smooth muscle Rho-kinase. The light chain phosphorylation site for Rho-kinase and MLCK is Ser^{19} and thus the effect of phosphorylation by the two kinases on actin-activated ATPase activity is the same. The critical question is whether myosin is phosphorylated by Rho-kinase under physiological conditions? An earlier report (19) showed that a constitutively-active fragment of Rho-kinase could induce contraction of Triton X-100-skinned portal vein via the phosphorylation of myosin, but to date this has not been demonstrated using the native, or full-length enzyme. Using Rho-kinase inhibitors the important role of Rho-kinase in smooth muscle function recently was shown (20) but these effects could result from phosphorylation of MYPT1 or myosin, or both. Our data with the native enzyme from smooth muscle suggests that direct phosphorylation of myosin by Rho-kinase is feasible. The $k_{cat}$ values for MLCK and Rho-kinase also are comparable. Presumably, if this occurs in situ it would be under conditions where MLCK activity is reduced, possibly at low Ca^{2+} concentrations. A signal to activate Rho-kinase obviously is required. The cascade involving translocation of RhoA to the membrane (47) and subsequent recruitment of Rho-kinase has the problem that myosin or MYPT1 may not be accessible to a membrane-bound kinase. A cytosolic active form of Rho-kinase may be more effective and it is possible that arachidonic acid activates the cytosolic pool of Rho-kinase.

In summary, a Rho-kinase of chicken gizzard smooth muscle has been isolated and shown to be a ROKα isoform. It exhibits a broad substrate specificity, but two good substrates (based on phosphorylation rates) are the MP subunit, MYPT1 and myosin. Thio phosphorylation of MYPT1 causes inhibition of the MP holoenzyme and phosphorylation of the myosin light chains on Ser^{19} increases the actin-activated ATPase of myosin. $k_{cat}$ values for MLCK and Rho-kinase with myosin as substrate are comparable. An interesting finding was that arachidonic acid activated Rho-kinase independent of RhoA and it is suggested that this activation is a possible physiological mechanism.
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