Exchange of the Actin-bound Nucleotide in Intact Arterial Smooth Muscle*

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The actin-bound ADP was separated from cytoplasmic nucleotides by treatment of intact arterial smooth muscle with 50% ethanol. In $^{32}$P-labeled smooth muscle the actin-bound ADP and phosphate readily exchanged with the cytoplasmic $\gamma$-P$_{32}$P|ATP; the specific radioactivity of actin-bound ADP was equal to that of the $\beta$-phosphate of cytoplasmic ATP and the specific radioactivity of actin-bound phosphate was equal to that of the $\gamma$-phosphate of cytoplasmic ATP. In contrast, the exchange of the actin-bound ADP in skeletal muscle was very slow. The presence of cytoplasmic ATP was required for the exchange of the actin-bound ADP and phosphate; if ATP synthesis was inhibited the exchange was also inhibited. The extent of exchange was reduced in muscles contracted by histamine or K$^+$, and stomach. The data indicate a dynamic state of actin in smooth muscle. The data also suggest that polymerization-depolymerization of actin is part of the contraction-relaxation cycle of smooth muscle.

Recently, Mehta and Gunst (5) and Jones et al. (6) reported the presence of G-actin in smooth muscle, based on the method of DNase I inhibition and phalloidin staining, respectively. We attempted to get direct evidence for the existence of G-actin in smooth muscle, based on the method of DNase I inhibition and phalloidin staining, respectively. We attempted to get direct evidence for the existence of G-actin in smooth muscle, based on the method of DNase I inhibition and phalloidin staining, respectively. We attempted to get direct evidence for the existence of G-actin in smooth muscle, based on the method of DNase I inhibition and phalloidin staining, respectively. We attempted to get direct evidence for the existence of G-actin in smooth muscle, based on the method of DNase I inhibition and phalloidin staining, respectively. We attempted to get direct evidence for the existence of G-actin in smooth muscle, based on the method of DNase I inhibition and phalloidin staining, respectively. We attempted to get direct evidence for the existence of G-actin in smooth muscle, based on the method of DNase I inhibition and phalloidin staining, respectively. We attempted to get direct evidence for the existence of G-actin in smooth muscle, based on the method of DNase I inhibition and phalloidin staining, respectively. We attempted to get direct evidence for the existence of G-actin in smooth muscle, based on the method of DNase I inhibition and phalloidin staining, respectively.

These equations form the rationale of our work, which reveals a rapid exchange of the G-actin bound-ATP in intact smooth muscle. Such an exchange is very slow in skeletal muscle.

EXPERIMENTAL PROCEDURES

$^{32}$P Labeling of Arterial Smooth Muscles—Porcine carotid arteries were obtained from the local slaughterhouse from freshly killed pigs. The arteries were transported in ice-cold physiological salt solution (PSS), containing in mM: 130 NaCl, 4.7 KCl, 1.2 MgSO$_4$, 2.5 CaCl$_2$, 0.03 CaEDTA, 14.9 NaHCO$_3$, and 5.5 glucose, into the laboratory, cleaned, mounted in special chambers, and resting tension adjusted (10). The arterial muscles were regenerated by incubation in 70 ml of PSS, at 37 °C under 95% O$_2$ and 5% CO$_2$ for 2 h. The muscles were ~4.5 cm long, 0.4–0.5 cm wide, and their wet weight was 0.4–0.5 g. At the end of the incubation, carrier-free inorganic $^{32}$P orthophosphate ($^{32}$P$_{i}$), 0.5–1.0 mCi, was added to the bath (containing 2 muscles) and $^{32}$P labeling of the muscles was continued until the required time. $^{32}$P$_{i}$ was removed from the extracellular space of the muscle by quick washings with PSS 15 times, each washing with 70 ml of PSS. Then the muscle was blotted gently, cut in the middle, one half was frozen in liquid nitrogen while the other half was dropped into 50% ethanol of 0 °C. Smooth muscles from pregnant uteri (rat and pig), urinary bladder (rabbit and pig), and stomach (rat and pig) were prepared as described (11) and labeled with $^{32}$P$_{i}$ by the same procedure as used for the arterial smooth muscle.

$^{32}$P Labeling of Contracted Muscles—After the 2-h recovery incubation, the PSS was exchanged for 70 ml of contracting solution containing either 0.1 mM histamine or a K$^+$-stimulating solution (in mM: 35 NaCl, 100 KCl, 1.2 MgSO$_4$, 2.5 CaCl$_2$, 0.03 CaEDTA, 14.9 NaHCO$_3$, and 5.5 glucose), and the muscle was allowed to develop tension for 10 min. Then the bath was exchanged for $^{32}$P$_{i}$ containing contracting solution and $^{32}$P labeling was allowed to proceed for 20 min (cf. Fig. 5). The muscles were washed with 70 ml of contracting solution 15 times, then cut and fixed in liquid nitrogen or 50% ethanol solution.

Workup of the Muscles—The frozen muscle was pulverized (10) and the powder from 4 half-muscles was extracted with 25 ml of 1.5% PCA in an ice bath for 2 min. After a brief high-speed centrifugation, 0.5-ml aliquots, in duplicate, were taken from the supernatant for determination of the specific radioactivity of phosphocreatine (PCr) (12), whereas the remainder of the extract was poured into an equivalent 10.0 v/v KOH solution to neutralize it to pH 7.5–8.0. After overnight standing in ice, the perchlorate precipitate was removed by centrifugation; the supernatant was concentrated by the SpeedVac and used for Dowex-1 chromatography.

In the ethanol procedure, each 4 half-muscle was extracted with 40 ml of 50% ethanol in ice overnight. The next morning the muscles were

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† The abbreviations used are: PSS, physiological salt solution; PCA, perchloric acid; PCr, phosphocreatine; $^{32}$P$_{i}$, inorganic $^{32}$P$_{i}$orthophosphate.
cut with scissors, each half into 3 pieces, and extracted four more times, each extraction with 40 ml of 50% ethanol in ice bath with stirring for 1 h. The absorbance and radioactivity of each extract were recorded. The first PCA extract was neutralized with 10 N KOH solution and prepared for Dowex-1 chromatography, as described before.

**Dowex-1 Chromatography**—The resin, AG-1 X8 200–400 mesh (Bio-Rad) in the chloride form, was washed twice, with 1.0 N NaOH and 1.0 N HCl, so that no UV absorbing material was eluted from the washed resin. The sample was dissolved in 1 ml of cold distilled water and clarified by high-speed centrifugation. The sample, containing 10 mg of protein, was layered on top of a column 15 cm long and 1 cm in diameter and eluted stepwise with 40 ml of 0.01 N HCl (elutes nucleosides, PCr, AMP, and other nucleotide monophosphates), 55 ml of 0.025 N HCl (elutes inorganic phosphate, ADP, and other nucleotide diphosphates), and 45 ml of 0.12 N HCl (elutes ATP and other nucleotide triphosphates) (modified from Ref. 13). The flow rate was 0.75 ml per min, 3.0-ml fractions were collected and the absorbance was recorded at 260 and 280 nm. Since in the muscle adenine nucleotides are predominant, the column actually separates AMP, ADP, and ATP, and PCr (Fig. 1). In addition to these compounds, the column also separates Pcr.

**Determination of Muscle Weight**—After the last extraction, the muscle residue in 1.5% PCA was stirred with 20 ml of 0.1 N NaOH at room temperature for a day. By this procedure, all muscle proteins are solubilized with the exception of connective tissue proteins, mainly collagen (14). The noncollagenous proteins in the supernatant were quantified by the modified biuret procedure (15) and converted in terms of weight of arterial muscle. This factor was determined by extracting known weight of arteries with 0.1 N NaOH under the same conditions.

**Pathways for the Incorporation of $^{32}$P into ATP**—The $^{32}$P, added into the bath of the muscle, permeates into the intracellular water and condenses with ADP (produced by the basal metabolism), to synthesize $^{[32P]}$ATP,

$$[^{32}P] + ADP \rightarrow [\gamma-^{32}P]ATP$$

(Eq. 3)

ADP is also produced through the creatine kinase catalyzed reaction,

$$[^{32}P]ATP + ADP \rightarrow [\gamma-^{32}P]ADP + ATP$$

(Eq. 4)

This reaction is very fast and under all conditions the specific radioactivity of $[^{32}P]ATP$ is equal to that of $[^{32}P]PCr$.

The ATP labeled at the $\gamma$-position is also labeled at the $\beta$-position, through the reaction catalyzed by the adenylate kinase,

$$[^{32}P]ATP + AMP \rightarrow [\beta-^{32}P]ADP + AMP$$

(Eq. 5)

and through the regular ATP synthesis,

$$[^{32}P] + [\beta-^{32}P]ADP \rightarrow [\gamma,\beta-^{32}P]ATP$$

(Eq. 6)

Labeling of ATP both at the $\gamma$ and $\beta$-positions is somewhat slower than labeling at the $\gamma$-position alone. We found complete double labeling after 2 h of incubation of the muscle with $[^32]P$. Double labeling of ATP was evidenced by the ratio of 2.0 for the specific radioactivity of $[^{32}P]ATP$ over the specific radioactivity of $[^{32}P]PCr$.

Under the conditions used here, the labeling of the $\alpha$-phosphate of ATP was very slow. We have found 5–8% labeling in muscles incubated with $[^32]P$, at 37 °C for 15 h. Labeling of $\alpha$-phosphate of ATP was evidenced by isolating $[^{32}P]$AMP with the Dowex-1 chromatography.

**Determination of the Exchange of the Actin-bound ADP**—In the intact muscle actin appears in the F-form with ADP as its bound nucleotide, the specific radioactivity of the $\beta$-phosphate of the cytoplasmic ATP is the reference for the exchange of the actin-bound ADP. Since short incubation of arterial muscle with $[^32]P$, labels only the $\gamma$ and $\beta$-phosphates of ATP, the specific activity of the $\beta$-phosphate of the cytoplasmic ATP can be calculated from the specific activity of the double labeled ATP minus the specific radioactivity of $[^{32}P]PCr$, namely the specific radioactivity of $[^{32}P]PCr$ equals that of the $\gamma$-phosphate of ATP (12). Thus,

$$S.A. of \beta$-phosphate of ATP = S.A. of $[^{32}P]ATP - S.A. of $[^{32}P]PCr$$

(Eq. 8)

and the exchange of the actin-bound ADP is expressed as,

**Percentage exchange (ADP)**

$$= \left(\frac{S.A. of actin-bound ADP}{S.A. of \beta$-P of cytoplasmic ATP}\right) \times 100$$

(Eq. 9)

The specific radioactivity of ATP and ADP was calculated from the counts/min values of the top five fractions of their peaks in the Dowex-1 chromatograms (cf. Figs. 1 and 3) and the absorbance of these fractions at 260 nm, using a molar absorption coefficient of 14.2 × 10$^4$ for adenine nucleotides at acidic pH (16).

As will be shown, the actin-bound phosphate also exchanges and the specific radioactivity of $[^{32}P]PCr$ is the reference for this exchange. Thus, the exchange of the actin bound $[^{32}P]$ is expressed as,

**Percentage exchange (P$_i$)**

$$= \left(\frac{S.A. of actin-bound P$_i$/S.A. of P$_{cr}$}{S.A. of $[^{32}P]PCr$}\right) \times 100$$

(Eq. 10)

The specific radioactivity of actin-bound $P_i$ was calculated from the counts/min values of the top three fractions of its peaks in the Dowex-1 chromatograms (cf. Fig. 3) and the absorbance of the phosphomolybdate complex at 720 nm, determined according to Rockstein and Herron (17).

**Actin—**Actin was prepared from rabbit skeletal muscle (15), polymerized with 0.1 M NaCl, and purified by ultracentrifugation at 105,000 × g for 3 h (modified from Ref. 18). The F-actin pellet was rinsed with distilled water twice, to remove salts, and dissolved by a Teflon-glass homogenizer in 0.4 mM ATP, pH 7.5, 10–12 mg of protein/ml, 0 °C. (By measuring the protein concentration of the actin solution before ultracentrifugation and of the supernatant after ultracentrifugation the protein content of the F-actin pellet could be estimated.) Upon prolonged homogenization (50–70 strokes) the actin was completely depolymerized and when centrifuged at 144,000 × g for 1 h only a small sediment (presumably F-actin) appeared. When the concentrated G-actin was treated with 20 mg of AG-1 X 800–400 mesh resin per ml of actin solution, 4 °C, for 5 min to remove unbound ATP, the G-actin polymerized. For this reason, no resin treatment was performed. The G-actin solution was divided into two parts, 1/10th volume of 1.0 M NaCl and 0.01 M MgCl$_2$, solution was added to one part to form F-actin, while the other part remained G-actin. After the preparation of G-actin and F-actin, the experiments started immediately and all treatments were carried out in an ice bath.

**Miscellaneous**—Data were expressed as mean ± S.E. Radioactivity was measured by liquid scintillation counting. $[^{32}P]$ was obtained from ICN.

**RESULTS**

**Separation of Actin-bound Nucleotides from Cytoplasmic Nucleotides in Intact Smooth Muscle**—Verhoeven et al. (19) separated the free and actin-bound nucleotides of platelets in 56% ethanol containing 6.7 mM EDTA. Applying their procedure to arterial smooth muscle, we have found no need for EDTA and reduced the ethanol concentration to 50% to increase the solubility of nucleotides and inorganic salts of muscle in the aqueous ethanol.

Fig. 2 shows the extraction of nucleotides and radioactivity...
from 32P-labeled arterial muscles upon treatments with 50% ethanol and subsequently with 1.5% PCA. In the first extract 46.1% of the total nucleotide absorbance at 260 nm and 58.8% of the total counts were solubilized. In the second extract these numbers decreased to 10.5 and 12.2%, respectively, and in the third extract they decreased to 3.0 and 3.6%, respectively. The remaining percentage absorbance was 0.5 in the fourth extract and zero in the fifth and sixth extracts; small amounts of counts were observable in these extracts, approaching the zero level. At this stage, the solvent was changed to 1.5% PCA; this resulted in the appearance of 30% of the total absorbance and 19.2% of the total counts, whereas the second PCA extraction yielded 9.9% of the absorbance and 4% of the counts. Accordingly, PCA extracted from the ethanol-washed muscle about 40% of the total absorbance at 260 nm and about 23% of the total counts. As will be described, this absorbance and counts belong to the actin-bound nucleotide and phosphate in the muscle. In comparing the percentage absorbance with the percentage counts extracted, it should be noted that all the absorbance refers to nucleotides, but the counts include compounds, which do not absorb in the U.V., e.g. the intermediates of glycolysis.

The Stability of Pure Actin in 50% Ethanol—Straub (1) observed that actin could be precipitated with ethanol without loss of activity. In our experiments, 50% ethanol did not precipitate G-actin or F-actin, and after the ethanol was removed G-actin solutions retained their ability to polymerize and F-actin solutions remained viscous. In our attempt to measure the effect of 50% ethanol on the bound nucleotide content of actin, we rediscovered (7) that ATP does not permeate through standard dialysis membranes. Subsequently, the following protocol was used for studying the stability of the actin-bound nucleotide in 50% ethanol.

To purified G-actin or F-actin, 10.5–11.5 mg/ml, in an ice bath, equal volume of cold absolute ethanol was added in small increments. The solutions were kept in the ice bath for 1 day. Control G-actin and F-actin solutions were prepared by adding an equal volume of cold distilled water to the actin solutions. Aliquots, 1 ml, were removed at time intervals of 3, 6, 12, and 24 h from the ethanol treated and control actin solutions and dialyzed against 15 liters of distilled water (G-actin) or 15 liters of 0.1 M NaCl and 1 mM MgCl₂ solution (F-actin) in the cold room for 1 h. After the dialysis, the ethanol content of the treated samples was measured with alcohol dehydrogenase (Sigma ethanol diagnostic test) and it was reduced from the original 50% ethanol to less than 0.5% ethanol. To quantify their nucleotide content, the G-actin solutions were polymerized by the addition of 1/10th volume of 1.0 M NaCl and 0.01 mM MgCl₂ solution and subjected to ultracentrifugation, along with the dialyzed F-actin samples, at 144,000 × g for 1 h. Virtually all the actin protein, 91–97%, was sedimented under these conditions. The supernatant was removed and the pellet was rinsed with distilled water; the pellet was dissolved by homogenization in 3.0 ml of 0.2 M NaOH, then the proteins precipitated with excess perchloric acid. After centrifugation, the absorbance of the supernatant was measured at 260 and 280 nm, the pellet was dissolved in 1.0 ml of 1.0 N NaOH and its protein content measured by the biuret method. The bound nucleotide content of the 50% ethanol-treated G-actin or F-actin did not differ from that of the untreated G-actin or F-actin. Furthermore, the bound nucleotide content did not decrease as a function of time during the 1-day incubation. The bound nucleotide content was in the range of 0.8–0.9 μmol of nucleotide per 42 mg of protein (n = 3). These results show that purified G-actin and F-actin retained their bound nucleotide during 1-day treatment with 50% ethanol.

Analysis of the Actin-bound Nucleotide—Fig. 3 shows the Dowex-1 chromatography profile of actin-bound nucleotide, isolated from arterial muscle by ethanol treatment. There are two nucleotides present, ADP and ATP with an approximate ratio of 7 to 1. There are three radioactive peaks; the first comes from P₃, the second from ADP, and the third from ATP. (As described in the next paragraph, the P₃ peak represents the actin-bound phosphate.) The specific radioactivity, in terms of cpm/μmol, was: 9.53 ± 0.21 × 10⁶ for P₃, 9.76 ± 0.75 × 10⁶ for ADP, and 20.10 ± 1.79 × 10⁶ for ATP. The specific radioactivity of the cytoplasmic ATP, from the same muscle, was 19.85 ± 0.63 × 10⁶, and that of PCr was 10.01 × 10⁶. From the last two data, using Equation 8, the specific radioactivity of β-phosphate of ATP was calculated to be 9.84 × 10⁶ cpm/μmol. By comparing the specific radioactivity of actin-bound ADP with that of the β-phosphate of cytoplasmic ATP, and comparing the specific radioactivity of actin-bound ATP with that of the cytoplasmic ATP, it is evident that the actin-bound nucleotide was completely exchanged with the γ(β-32P)ATP in the intracellular water. The data also show that during the 2-h incubation of the muscle with 32P, the specific radioactivity of β-phosphate of the cytoplasmic ATP reached the specific radioactivity of its γ-phosphate. Furthermore, the specific radioactivity of the actin-bound phosphate reached the specific radioactivity of PCr.

Actin-bound Phosphate—The following data suggest the existence of actin-bound phosphate. The first 50% ethanol extract of arterial muscle (Fig. 2) is loaded with 32P, due to the complete hydrolysis of cytoplasmic γ(β-32P)ATP. (This was shown by Dowex-1 chromatography.) Through the successive ethanol
extractions, the \(^{32}\)P\(_i\) is removed from the muscle completely (Fig. 2). However, when the residue is extracted with PCA the \(^{32}\)P\(_i\) reappears (Fig. 3).

In muscles incubated with \(^{32}\)P\(_i\), for a short time, the specific radioactivity of the actin-bound \(P_i\) differed greatly from that of the actin-bound ADP. For instance, with 20 min incubation the specific radioactivity of actin-bound \(^{32}\)P\(_i\)ADP was 1.87 \(\times 10^6\) cpm/\(\mu\)mol, the specific radioactivity of the actin-bound \(^{32}\)P\(_i\)ADP was 5.01 \(\times 10^5\) cpm/\(\mu\)mol, and the specific radioactivity of \(^{32}\)P\(_i\)PCr was 5.79 \(\times 10^6\) cpm/\(\mu\)mol. Thus, the actin-bound \(P_i\) resembled PCr and not actin-bound ADP. Since the specific radioactivity of \(^{32}\)P\(_i\)PCr equals that of \(\gamma,\beta\)-\(^{32}\)P\(_i\)ATP during the polymerization of actin (Equation 2). The concentration of the actin-bound \(P_i\) was 60–85% of that of the actin-bound ADP. In vitro studies showed that the actin-bound \(P_i\) dissociates slowly, whereas the actin-bound ADP does not dissociate in practical terms (21–23). Thus, the reduced phosphate content of actin in the muscle, relative to its ADP content, can be explained by the slow dissociation of the phosphate from actin during the prolonged 50% ethanol washing of the arterial muscle.

Quantification of the Actin-bound Nucleotide—From the absorbance of the PCA extract and the protein content of the PCA residue, we calculated 1.11 \(\pm\) 0.10 \(\mu\)mol of nucleotide/g of wet muscle weight (\(n = 35\)). All this nucleotide was fully exchanged when resting muscle was incubated with \(^{32}\)P\(_i\), for 45–60 min. With a molecular mass of 42,000 daltons (20), the actin content was 46.6 mg/g muscle in good agreement with the reported actin content of carotid arteries, 48.5 mg/g (20). ADP comprised 85–88% of the bound nucleotide, while ATP amounted to 12–15%.

Exchange of the Actin-bound Nucleotide in Resting Muscle—Fig. 4 shows the percentage exchange as a function of time for porcine carotid arterial smooth muscle and rat skeletal muscle. In the case of the smooth muscle, no reasonable measurements could be performed in the first 15 min of the \(^{32}\)P\(_i\) incubation. During this period the \(^{32}\)P\(_i\) permeates into the cytoplasm (a slow process), followed by the synthesis of \(^{32}\)P\(_i\)-labeled ATP, and only then can an exchange of the actin-bound ATP take place. At the 15-min incubation the exchange was 53%, raising to 81% at 20 min, and 95% at 30 min. Subsequently, the exchange remained at 100% level up to 6 h of incubation.

In contrast to the rapid exchange in smooth muscle, the exchange in rat skeletal muscles was very slow. The exchange was followed in hourly intervals and reached about 15% after 3 h of incubation. Not shown are results with frog leg skeletal muscles, incubated at 25 °C (at 37 °C frog muscles denature) that also exhibited a slow exchange. These data are in good agreement with the results of Martonosi et al. (4) who discov-
affected in the poisoned muscles although the cytoplasmic ATP was degraded to AMP and IMP. Thus, in the three cases described before the bound nucleotide content was 0.88, 0.91, and 1.05 μmol of nucleotide/g of wet muscle weight. This proves the idea that the bound-ADP is not available to cytoplasmic enzymes, which participate in ATP turnover in muscle (3).

*Ca*\(^{2+}\) Is Not Involved in the Exchange—Arterial muscles were incubated in Ca\(^{2+}\)-free PSS, which contained in addition 1.0 mM EGTA, for 2 h and then with \(^{32}\)P in Ca\(^{2+}\)-free PSS and EGTA for 45 min. The labeled muscles were washed with Ca\(^{2+}\)-free PSS and EGTA 7 times and with PSS 8 times. The actin-bound ADP and P\(_i\) were fully exchanged in such muscles, the same way as they exchanged in control muscles treated with normal PSS for the same time.

**Analysis of the Perchloric Acid Extract**—Two major nucleotide peaks appeared on the Dowex-1 chromatography of the PCA extract from \(^{32}\)P-labeled arterial muscle (Fig. 1), the first corresponded to ADP and the second to ATP. Because of the total ADP in PCA extract of smooth muscle (or any other muscle) is essentially the actin-bound ADP (25, 26), from results of this Dowex-1 chromatography the percentage exchange of actin-bound ADP could be calculated. Thus, from the specific activities of cytoplasmic ATP, PCr, and actin-bound ADP, using Equations 8 and 9, the percentage exchange of the actin-bound ADP was found to be 94%. A similar value, 99%, was obtained for the other half of the same muscle, which was analyzed by the ethanol procedure.

**Other Smooth Muscles**—Table II compares the exchange of actin-bound ADP and P\(_i\) in arterial muscle with those of muscles from rat and porcine uterus (pregnant), from rabbit and porcine urinary bladder, and from rat and porcine stomach. The overall data show a complete or nearly complete exchange in each of these smooth muscles. The somewhat larger standard error for stomach and bladder muscles reflects the difficulties in preparing pure smooth muscle from these tissues in larger quantities.

**DISCUSSION**

We confirm and extend the finding of Mehta and Gunst (5) and Jones et al. (6) on the existence of G-actin in smooth muscle. With \(^{32}\)P labeling we show that all actin molecules in porcine carotid artery readily exchange their bound ADP and P\(_i\). Because of the tight coupling between actin-bound nucleotides and actin polymerization (3), the observed exchange reflects a polymerization-depolymerization, actin-repolymerization cycle of actin. Applying the \(^{32}\)P method to uterine, urinary bladder, and stomach smooth muscles from various sources, we find a similar exchange of the actin-bound ADP and P\(_i\). Under the same conditions, rat and frog skeletal muscles show only a small exchange. These data when combined with those of Mehta and Gunst (5) and Jones et al. (6) obtained on canine tracheal smooth muscle, suggest that the reversible polymerization of actin is a basic property of every smooth muscle.

The present study establishes a new procedure for the separation of cytoplasmic nucleotides from actin-bound nucleotides at the level of intact smooth muscle using 50% ethanol. At this ethanol concentration, the muscle is readily permeabilized at 0 °C and thus releases all its free organic and inorganic constituents, while actin remains in its native state and thereby keeps its bound nucleotide. Purified G-actin and F-actin also retain their bound nucleotide upon prolonged ethanol treatment. On the other hand, myosin is irreversibly denatured even with 20% ethanol in muscle extract (27), thus it seems unlikely that at the 50% ethanol concentration used in this work any nucleotide would have remained bound to myosin. Furthermore, smooth muscle contains only 100 nmol of myosin heavy chain (20), as compared with 1,100 nmol of actin; therefore, myosin cannot be responsible for the exchange of 1,100 nmol of bound nucleotides, demonstrated in this study.

The results of this work confirm the generally accepted view that actin is in the F-form in intact muscle, because liquid nitrogen frozen and subsequently PCA extracted arterial muscle yields ADP as the actin-bound nucleotide. The rapid equilibration of F-ADP with the cytoplasmic ATP in smooth muscle can be explained by the following:

\[
\text{F-ADP} + [\gamma, \beta, \text{AMP}] \text{ATP} \rightarrow [\gamma, \beta, \text{AMP}] \text{ATP} + \text{ADP} \quad (\text{Eq. 11})
\]

\[
[\gamma, \beta, \text{AMP}] \text{ATP} \rightarrow [\gamma, \beta, \text{AMP}] \text{ADP} + ^{32}\text{P}_i \quad (\text{Eq. 12})
\]

The isolation of the [\gamma, \beta, \text{AMP}]ATP intermediate from \(^{32}\)P-labeled smooth muscle (Fig. 3) supports this mechanism of the exchange. The scheme does not involve the two distinct steps found in vitro during the hydrolysis of ATP associated with actin polymerization (21), namely the F-ATP and F-ADP-P\(_i\) intermediates. However, our data reveal the existence of F-[\beta, \text{AMP}]ADP, \(^{32}\)P\(_i\) intermediate in the muscle (Fig. 3).

These data indicate the dynamic state of actin in smooth muscle. They also support the suggestion of Mehta and Gunst (5) that contractile activation of smooth muscle is associated with enhanced polymerization of actin, a basic requirement for force generation in muscle. Furthermore, the data suggest that the reversible polymerization of actin is part of the contraction-relaxation cycle of smooth muscle.

Since smooth muscle contains over 1 μmol of actin per g, the reversible polymerization of actin coupled to ATP hydrolysis, followed by ATP resynthesis, has a considerable energy requirement. All evidence suggests that the actin protein itself generates the energy through the “mechanochemistry” of actin production.
(3). Since the actin-bound nucleotide is not available for enzymes involved in ATP turnover, the transformation of the actin polymer to actin monomer may provide the energy for ATP synthesis (3).

It seems likely that actin-binding proteins are controlling the actin dynamics in smooth muscle, well established in non-muscle cells (28, 29). The role of profilin, thymosin β-4, in the control of actin nucleotide exchange has been described in vitro (30, 31). Other actin-binding proteins, ADF/cofilin (32), the Arp2/3 complex (33), or leiomodin and tropomodulin (34), may be regulators of actin filament assembly in animal cells including smooth muscle (34).

Smooth muscle, unlike skeletal or cardiac muscle, contains a large excess of actin over myosin. The physiological role of this actin surplus is not known. Recent trends in smooth muscle research point to a functional role of the cytoskeleton, the main component of which is actin (35). Analysis of 32P incorporation into the actin-bound nucleotide and phosphate in intact live muscle may help to find the role of actin in cytoskeleton function.

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