Adenylate Cyclase in Skeletal Muscle

KINETIC PROPERTIES AND HORMONAL STIMULATION*

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SUMMARY

Adenylate cyclase was studied in plasma membranes prepared from rabbit skeletal muscle. Such preparations represented an increase in specific activity of 10- to 20-fold over the whole homogenate with a yield of activity of approximately 30%. Various parameters, such as phase contrast microscopy, "marker" enzyme activities, and chemical composition, suggested that the preparation constituted sarcolemma in a high degree of purity. The $K_m$ for substrate (MgATP) was 0.3 to 0.5 mM. The $K_i$ for Mg$^{2+}$ was 3 to 5 mM. Binding of Mg$^{2+}$ to a second site resulted in increased reactivity of the catalytic site for substrate. Stimulation by fluoride resulted from an increase in maximal velocity; the $K_m$ for Mg$^{2+}$ and the $V_{	ext{max}}$ for substrate were not appreciably altered. The effect of F$^-$ was markedly temperature-sensitive and partially irreversible. Fluoride-stimulated activity was particularly sensitive to inhibition by pyrophosphate and this inhibition was competitive with respect to ATP ($K_i$, 0.43 mM). Catecholamines stimulated the enzyme in a typical $\alpha$ adrenergic fashion. The prominent kinetic effect of epinephrine was (like F$^-$) to increase reaction velocity without affecting affinity for Mg$^{2+}$ or ATP. The regulation of adenylate cyclase in skeletal muscle may be classified as a "V" allosteric system since metal ions, F$^-$, and epinephrine all result in increased maximal velocity of the enzyme reaction.

Much evidence exists that adenylate cyclase is located primarily, but not exclusively, in plasma membranes of cells of most tissues. The enzyme has not been extensively studied in skeletal muscle although it was shown to be present in this tissue some years ago (1). The formation of cyclic adenosine 3',5'-monophosphate by a microsomal fraction of rabbit skeletal muscle was reported by Borel and Drabkin (2). Rabinowitz et al. (3) have reported that the enzyme present in homogenates of this tissue was distributed largely in mitochondrial and microsomal fractions. Methods have been available for some time for the isolation of skeletal muscle sarcolemma (4, 5), but these preparations have not been examined for adenylate cyclase activity.

EXPERIMENTAL PROCEDURE

Uniformly labeled $[^{14}C]$ATP (418 to 462 mCi per mmole), cyclic [H]AMP (24 Ci per mmole), [alpha-32p]ATP (1.76 to 10.9 Ci per mmole), and $[^{45}C]$Cl (20.4 mCi per mg) were obtained from New England Nuclear. Ethanol was removed from the $[^{14}C]$ATP solutions under vacuum. Solutions of labeled ATP were diluted with unlabeled nucleotide to the desired specific activity and with water to the desired concentrations. Cyclic [8-$[^{3}H]$AMP (33.6 mCi per mmole) was purchased from Schwartz BioResearch. Unlabeled nucleotides, 2-phosphoenol pyruvate (trisodium salt), pyruvate kinase (rabbit muscle), phenylephrine hydrochloride, and ouabain (strophanthin G) were obtained from Calbiochem. Epinephrine (L-adrenaline bitartrate) was obtained from K and K Laboratories, Brooklyn, New York; isoproxynondrenaline-HCl from Winthrop Laboratories, New York; noradrenaline (L-arterenol-beta-tartrate) and carbachol (carbamylcholine chloride) from Mann; DL-propranolol from Ayerst Laboratories, Montreal; insulin (bovine pancreas, 24 i.u. per mg) from Sigma; and Amanad-P reagent from Burdick and Jackson Laboratories, Muskegon, Michigan. Prostaglandins E$_1$, E$_2$, F$_{12a}$, F$_{2a}$, and A$_2$ were supplied through the courtesy of Dr. John Pike of The Upjohn Company.

Methods

Isolation of Plasma Membranes—Plasma membranes were isolated from rabbit skeletal muscle by a modification of the method of Kono and Colowick (4). All procedures were carried out at 4º. Fresh leg muscle from rabbits in 5-g portions was
following centrifugation, an aliquot (1.0 ml) of the supernatant was assayed for inorganic phosphate by the method of Taussky and Shorr (8). Mg⁺-dependent ATPase was measured under similar conditions with the omission of NaCl and KCl. Ca⁺⁺-dependent ATPase was determined by incubating appropriate quantities (0.20 to 0.30 mg) of membrane protein with 50 mM Tris-HCl, pH 7.0, 5 mM ATP, and 5 mM CaCl₂ in a final volume of 1 ml for 10 to 20 min at 37°C. Phosphate was determined as described above. 5'-Nucleotidase was assayed by incubating muscle protein with 8 mM 5'-AMP, and 18 mM MgSO₄ in a final volume of 250 μl for 10 min at 37°C. Reactions were terminated by the addition of 1 ml of cold 3% trichloroacetic acid. After centrifugation, inorganic phosphate was determined in the supernatant by the Fiske and SubbaRow method (9). Acid phosphatase was estimated by determining inorganic phosphate released (9) following the incubation of muscle protein with 25 mM β-glycerol phosphate buffered at pH 5.0 with 50 mM acetic acid in a final volume of 200 μl for 20 min at 37°C. Pyrophosphatase was assayed according to the method of Nordlie and Arion (10); acid maltase by the method of Iles and van Hoof (11), cytochrome c oxidase as described by Cooperstein and Lazarew (12) and acetyl cholinesterase by the spectrophotometric method of Filman et al. (13).

Skeletal Muscle Adenylate Cyclase

Vol. 247, No. 9

minced with scissors and homogenized for 15 s in 5 volumes of 50 mM CaCl₂ in a Sorvall Omnimixer at maximal velocity. The homogenate was passed through a coarse nylon sieve (pore size 1 mm) and centrifuged for 10 min at 2,000 × g. The precipitate was washed twice by suspending to the original volume in 10 mM Tris-HCl, pH 8, and centrifuging at 2,000 × g for 10 min. Washing effectively removed soluble protein and superficially bound calcium. Preparations of washed particles were suspended in 5 volumes of 10 mM Tris-HCl, pH 8 (based on initial tissue weight), and centrifuged at 2,000 × g for 10 min. Routinely, incubation mixtures were chromatographed in isobutyric acid-95% ethanol (15:35) as the developing solvent (6). In experiments in which substrate concentrations greater than 0.3 mM were required, [α-³²P]ATP was used as substrate instead of [³²P]ATP. Following termination of the reaction by boiling, 50 μl of 0.25 M ZnSO₄ were added to each tube, followed by 50 μl of 0.25 M Ba(OH)₂. Precipitates were removed by centrifugation at 8,000 × g, and 150 μl of each clear supernatant were subjected to chromatography in the usual way (6). Barium-zinc precipitation removed more than 95% of the ATP, ADP, and 5'-AMP present; recovery of cyclic AMP ranged from 97 to 100% as determined by the addition of known amounts of the ³⁰Cl-labeled compound to control tubes.

Assay of Other Enzymes—The assay for Mg⁺⁺-dependent Na⁺, K⁺-stimulated ATPase contained 50 mM Tris-HCl, pH 7.5, 5 mM ATP, 5 mM MgCl₂, 100 mM NaCl, 20 mM KCl, and appropriate quantities of membrane protein (0.20 to 0.30 mg) in a total volume of 1 ml. The tubes were incubated for 10 min at 37°C. Ouabain when present was 0.4 mM. The reaction was terminated by the addition of 1 ml of cold 12% trichloroacetic acid, and, following centrifugation, an aliquot (1.0 ml) of the supernatant was assayed for inorganic phosphate by the method of Tauskay and Shorr (8). Mg⁺⁺-dependent ATPase was measured under similar conditions with the omission of NaCl and KCl. Ca⁺⁺-dependent ATPase was determined by incubating appropriate quantities (0.20 to 0.30 mg) of membrane protein with 50 mM Tris-HCl, pH 7.0, 5 mM ATP, and 5 mM CaCl₂ in a final volume of 1 ml for 10 to 20 min at 37°C. Phosphate was determined as described above. 5'-Nucleotidase was assayed by incubating muscle protein with 8 mM 5'-AMP, and 18 mM MgSO₄ in a final volume of 250 μl for 10 min at 37°C. Reactions were terminated by the addition of 1 ml of cold 3% trichloroacetic acid. After centrifugation, inorganic phosphate was determined in the supernatant by the Fiske and SubbaRow method (9). Acid phosphatase was estimated by determining inorganic phosphate released (9) following the incubation of muscle protein with 25 mM β-glycerol phosphate buffered at pH 5.0 with 50 mM acetic acid in a final volume of 200 μl for 20 min at 37°C. Pyrophosphatase was assayed according to the method of Nordlie and Arion (10); acid maltase by the method of Iles and van Hoof (11), cytochrome c oxidase as described by Cooperstein and Lazarew (12) and acetyl cholinesterase by the spectrophotometric method of Filman et al. (13).

Cyclic 3',5'-nucleotide phosphodiesterase was measured by incubating cyclic (³²P)AMP (5 mM, 2 μc per mg protein) with 25 mM Tris-HCl, pH 7.5, 2.5 mM MgSO₄, and protein in a final volume of 200 μl for 20 min at 30°C. Reactions were terminated by immersing the tubes in a boiling water bath, and, following removal of denatured protein by centrifugation, 150 μl of the supernatant fluid were chromatographed, with 1 mM ammonium acetate-95% ethanol (15:35) as the developing solvent (6). Areas of the chromatogram corresponding to 5'-AMP (identified by marker) were cut out and counted by scintillation spectrometry. Under the conditions of the assay there was no detectable conversion of 3'-AMP to adenosine.

Cyclic 2',3'-nucleotide phosphodiesterase was assayed by the method of Drummel et al. (14).

Measurement of Calcium Binding—Membrane protein (0.20 to 0.30 mg per ml) was incubated in a medium (final volume 2 ml) containing 50 mM Tris-maleate, pH 6.0, 5 mM MgCl₂, 5 mM ATP, and 0.1 mM ⁴⁰CaCl₂ (4000 to 6000 cpm per nmole) at 37°C. Reactions were terminated by filtering the mixtures through Millipore filters (0.45 μ, 25 mm). The filters were carefully washed with 5 ml of the above buffer, dried, and radioactivity was determined by scintillation spectrometry. Appropriate controls were run containing no ATP, no MgCl₂, or no protein. In the absence of membrane protein, retention of radioactivity on the filters was negligible. Calcium binding was calculated from the specific activity of added ⁴⁰CaCl₂ and the activity retained by the membrane protein.

Chemical Assays—Cholesterol was determined by the method of Zak et al. (15). Lipid phosphorus was determined by the procedure described by Bartlett (16); phospholipid was estimated by multiplying the lipid phosphorus value by 25. Sialic acid was determined by the method of Warren (17) following hydrolysis of membrane protein by the procedure of Svennerholm (18). Fluoride was estimated with the Amadac-F reagent (19). Protein determinations were made by the method of Lowry et al. (20).

RESULTS

Preliminary—The presence of ATPase in the membrane preparations necessitated the use of an ATP regenerating system to preserve substrate. Routinely, incubation mixtures were chromatographed in isobutyric acid-1 M ammonium hydroxide-0.1 M...
Effects of unlabeled cyclic adenine 3',5'-monophosphate and theophylline on degradation and formation of cyclic AMP

Incubation mixtures contained 40 mM Tris-HCl, pH 8.5, 8 mM F-, 18 mM MgSO4, and the phosphoenol pyruvate-lyase kinase regenerating system. Experiment 1, cyclic [8-14C]AMP (10,902 dpm) was present without labeled ATP. Experiment II, [U-14C]ATP (0.3 mM, 20 μCi/pmol) was the substrate; labeled cyclic AMP was absent. In each experiment, unlabeled cyclic AMP and theophylline were added as indicated. After 20 min at 37° the reaction mixtures were boiled, centrifuged, and chromatographed. Labeled cyclic AMP was then determined as described under "Methods."

| Additions | Experiment I: cyclic [8-14C]AMP dpm recovered | Experiment II: cyclic AMP formed | pmoles/min/mg |
|-----------|---------------------------------------------|---------------------------------|---------------|
| Control   | 10,724                                      | 395                             | 298           |
| Membrane protein (65 μg) | 10,724                                      | 395                             | 298           |
| Membrane protein + 0.5 mM cyclic AMP | 10,266                                      | 708                             | 208           |
| Membrane protein + 2.0 mM cyclic AMP | 10,475                                      | 664                             | 196           |
| Membrane protein + 13 mM theophylline | 4,703                                      | 467                             | 347           |
| Membrane protein + 13.4 mM theophylline | 9,455                                      | 387                             | 347           |

EDTA (125:75:2) (6) to test the effectiveness of the phosphoenol pyruvate-lyase kinase regenerating system used. This solvent system separates ADP from ATP more effectively than the 1 mM ammonium acetate-95% ethanol (15:35) solvent system (6). In the absence of the regenerating system, incubating standard amounts of membrane protein (75 μg) in the assay led to the destruction of up to 75% of the ATP, the radioactivity being distributed between ADP and 5'-AMP. The inclusion of 8 mM F- did not prevent the disappearance of ATP, but decreased the amount of 5'-AMP (with an increasing proportion of radioactivity in ADP). Addition of the phosphoenol pyruvate-lyase kinase regenerating system effectively maintained more than 90% of the ATP. Assay conditions under which the regenerating system may fail to conserve substrate have been discussed previously (6, 7). Recovery of cyclic AMP in the reaction mixtures was also examined. In a mock assay, cyclic [8-14C]AMP (10,902 dpm) was added to the incubation mixture and the tubes were incubated with the omission of ATP, and with the addition of unlabeled cyclic AMP or theophylline to prevent destruction of the labeled material. Table I, Experiment I, shows that when cyclic [8-14C]AMP was incubated in the presence of membrane protein (95 μg), at least 95% was destroyed. Radioactivity on the chromatogram was recovered in the area corresponding to 5'-AMP (4,740 dpm) and adenosine (6,287 dpm), a reflection of the presence of both cyclic 3',5'-nucleotide phosphodiesterase and 5'-nucleotidase. Recovery of the labeled cyclic AMP was increased to greater than 95% by the addition of unlabeled cyclic nucleotide (0.5 mM and 2 mM) (Table I). The addition of theophylline (1.3 mM and 13.4 mM) also prevented the destruction of the cyclic [8-14C]AMP, but was less effective than unlabeled cyclic AMP. Addition of unlabeled cyclic AMP to the reaction mixture greatly increased the synthesis of labeled cyclic nucleotide from [U-14C]ATP (Table I, Experiment II). Addition of theophylline (1.3 mM and 13.4 mM) appeared to decrease the formation of product from labeled ATP. In a separate experiment, it was shown that increasing amounts of theophylline in the presence of 2 mM cyclic AMP, indeed, led to a concentration-dependent decrease in adenylate cyclase activity; 50% inhibition was produced with 20 mM theophylline. Inhibition of adenylate cyclase activity by theophylline has also been observed in the toad bladder (21) and rat erythrocyte (22). In the standard assay, therefore, we have employed unlabeled cyclic nucleotide as the sole means of preserving the labeled product. Under conditions finally adopted (theophylline absent, 2 mM cyclic AMP present), adenylate cyclase activity was proportional to time and to protein content when measured in the presence or absence of 8 mM F- (Fig. 1). The pH dependence was examined from pH 6.0 to 10.5 with the use of combinations of β-glycerol phosphate, Tris-HCl, and 2-amino-2-methyl-1,3-propanediol (each at 40 mM) as buffers. The pH optimum both in the presence and absence of F- was 8.5, somewhat higher than that previously found for the cardiac enzyme (6).

Adenylate Cyclase in Skeletal Muscle of Several Organisms—Enzyme activity in whole homogenates of skeletal muscle from several species is shown in Table II. Activity in back leg muscle of rabbit and guinea pig was greater than in rat leg muscle, particularly in the presence of F-. Mammalian skeletal muscles were more active than frog gastronomus. Pigeon breast muscle was much more active than chicken breast muscle. The low activity in frog muscle when compared with mammalian tissues and the differences between breast muscle from chicken and pigeon suggest that adenylate cyclase may be richer in red fibers adapted for oxidative metabolism than in white muscle fibers. Because of ready availability and high activity, rabbit hind leg muscle was selected for all subsequent studies.

Sedimentation of Adenylate Cyclase and Preparation of Plasma Membranes—When homogenates were prepared with a blade homogenizer, it was found that 80% of the enzyme activity was present in the particulate fraction prepared by sedimenting at 2,000 × g and washing with dilute buffer (Table III, Fraction A).
Most of the activity remaining in the 2,000 \( \times g \) supernatant could be sedimented at 37,000 \( \times g \). Such high yields of activity in particles sedimenting at low gravitational forces suggested that adenylate cyclase might reside in the plasma membrane. Accordingly, attempts were made to isolate sarcolemma from this tissue. Procedures based on the method of McCollester (5) resulted in large losses of activity. Modification of the procedure of Kono and Colowick (4) (see under "Methods"), however, yielded a plasma membrane fraction with a high yield of adenylate cyclase activity (Table III, Fraction B). Extraction of the washed particles with LiBr resulted in an increase in specific activity of about 2-fold with a 78% yield of activity. Centrifugation in KBr (density 1.21) produced a membrane pellet with a yield of 37% of the total activity and an increase in specific activity of adenylate cyclase of about 15-fold. Purification of activity measured in the absence of F\(^-\) paralleled that measured in the presence of this anion (8 mM). The enzyme present in membranes was highly unstable. Storage of membrane preparations for 18 hours at \(-4^\circ\) or even at \(-80^\circ\) led to 50% loss of activity. From 50 to 80% of the activity survived lyophilization of membrane preparations; lyophilized powders, however, lost 50% of their activity during storage at \(-18^\circ\) for 1 week. Because of this, all experiments were performed with freshly prepared membranes.

Membrane Properties—A number of criteria were examined to ascertain the relative purity of the plasma membrane preparation. At each step of the procedure, fractions were routinely examined by phase contrast microscopy. Photomicrographs of a washed particle and final membrane preparation are shown in Fig. 2. Cross striations are clearly visible in washed particle preparations (Plate A), whereas the membranes (Plate B) contain no cross striations and appear as empty, transparent, saclike structures analogous to those described by Kono and Colowick (4) and McCollester (5).

The membrane preparation contained a Mg\(^2+\)-dependent ATPase activity (Table IV, Addition A) which was further stimulated by the addition of 100 mM Na\(^+\) and 20 mM K\(^+\) (Table IV, Addition B). This Na\(^+\), K\(^+\)-stimulated Mg\(^2+\)-ATPase was partially inhibited by the addition of 0.4 mM ouabain. The specific activity of this enzyme was greater than that observed by Peter (23) for rat sarcolemma, but less than that present in hamster skeletal muscle sarcolemma (24). The membrane preparation also possessed Ca\(^2+\)-stimulated ATPase activity which did not require Mg\(^2+\) (Table IV, Addition C). Ca\(^2+\)-stimulated ATPase has been reported previously to be present in the skeletal muscle sarcolemmal membranes (26). In addition, the membrane preparations were able to bind calcium in an ATP-dependent manner.

![Fig. 2. Phase contrast photomicrograph of (A) washed particles and (B) final membrane preparation.](http://www.jbc.org/issue/247/9/Skeletal-Muscle-Adenylate-Cyclase)

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**TABLE II**

Adenylate cyclase in skeletal muscle from several organisms

Portions of hind leg muscle from rat, guinea pig, and rabbit, the gastrocnemius from frog, and breast muscle from pigeon and chicken were homogenized in 5 volumes of 10 mM Tris-HCl, pH 8.0, for 10 s in a Sorvall Omnimixer. The homogenates were strained through a coarse nylon sieve (pore size 1 mm\(^2\)). Enzyme activity was determined in reaction mixtures containing 0.3 \(\mu\)M [U-\(14\)C]-ATP, 18 mM MgSO\(_4\), and 1 \(\mu\)M EGTA. F\(^-\) when present yielded a plasma membrane fraction with a high yield of adenylate cyclase activity (Table III, Fraction B). Extraction of the washed particles with LiBr resulted in an increase in specific activity of about 2-fold with a 78% yield of activity. Centrifugation in KBr (density 1.21) produced a membrane pellet with a yield of 37% of the total activity and an increase in specific activity of adenylate cyclase of about 15-fold. Purification of activity measured in the absence of F\(^-\) paralleled that measured in the presence of this anion (8 mM). The enzyme present in membranes was highly unstable. Storage of membrane preparations for 18 hours at \(-4^\circ\) or even at \(-80^\circ\) led to 50% loss of activity. From 50 to 80% of the activity survived lyophilization of membrane preparations; lyophilized powders, however, lost 50% of their activity during storage at \(-18^\circ\) for 1 week. Because of this, all experiments were performed with freshly prepared membranes.

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ATPase activities of skeletal muscle membrane preparation

Enzyme activities were measured as described under "Methods" with the various additions indicated. Values for Mg\(^{2+}\)-ATPase and Ca\(^{2+}\)-ATPase are the average of three separate preparations.

| Addition                | ATPase activity (µmoles Pi/mg protein/min) |
|-------------------------|------------------------------------------|
| A. 0.2 mM Mg\(^{2+}\)   | 3.0                                      |
| 0.5 mM Mg\(^{2+}\)      | 3.3                                      |
| 1.0 mM Mg\(^{2+}\)      | 4.0                                      |
| 2.0 mM Mg\(^{2+}\)      | 8.4                                      |
| 5.0 mM Mg\(^{2+}\)      | 10.3                                     |
| B. 5 mM Mg\(^{2+}\)     | 16.1                                     |
| 5 mM Mg\(^{2+}\), 100 mM Na\(^{+}\), 20 mM K\(^{+}\) | 24.1                                     |
| 5 mM Mg\(^{2+}\), 100 mM Na\(^{+}\), 20 mM K\(^{+}\), 0.4 mM ouabain | 20.9                                     |
| C. 0.05 mM Ca\(^{2+}\)  | 1.7                                      |
| 0.10 mM Ca\(^{2+}\)     | 1.7                                      |
| 0.20 mM Ca\(^{2+}\)     | 2.2                                      |
| 0.50 mM Ca\(^{2+}\)     | 2.9                                      |
| 1.00 mM Ca\(^{2+}\)     | 3.4                                      |
| 2.00 mM Ca\(^{2+}\)     | 5.2                                      |
| 5.00 mM Ca\(^{2+}\)     | 7.9                                      |

Calcium binding by skeletal muscle membrane preparation

Calcium binding was measured as described under "Methods" with the additions indicated. Values given are the means of three different membrane preparations.

| Additions                | Calcium bound (µmoles/mg protein) |
|--------------------------|-----------------------------------|
| None                     | 1.5, 1.0, 0.9                      |
| ATP (5 mM)               | 6.1, 5.2, 4.5                      |
| ATP + P\(_i\) (2 mM)     | 28.1, 42.1, 65.0                   |

Lipid composition of rabbit muscle membrane fraction

Determinations are described under "Methods." Molar quantities of cholesterol and phospholipid were calculated assuming molecular weights of 387 and 700, respectively.

| Membrane preparation | Cholesterol/mg protein | Phospholipid/mg protein | Sialic acid/mg protein |
|----------------------|------------------------|-------------------------|-----------------------|
| A                    | 0.11                   | 270                     | 0.34                  |
| B                    | 0.14                   | 360                     | 0.49                  |
| C                    | 0.15                   | 390                     | 0.51                  |

Fig. 3. Effect of metal ions on skeletal muscle adenylate cyclase. The substrate was 0.3 mM [U-\(^{14}\)C]ATP and 91 μg of membrane protein were used; P\(_i\) absent. Metal ion concentrations were varied as indicated.

Properties of Adenylate Cyclase

Effect of Metal Ions—In agreement with earlier studies on the cardiac enzyme (7), metal ions stimulated skeletal muscle adenylate cyclase when present at concentrations in excess of ATP. The effect of Mg\(^{2+}\), Mn\(^{2+}\), and Co\(^{2+}\) in the presence of 0.3 mM ATP is shown in Fig. 3. The K\(_s\) for Mg\(^{2+}\) was estimated to be...
Skeletal Muscle Adenylate Cyclase

Vol. 247, No. 9

ATP (mM)

**FIG. 4.** Effect of ATP concentration on adenylate cyclase with three fixed Mg\(^{2+}\) concentrations. \([\alpha-\sp{32}P]ATP\) was used as substrate; concentrations of Mg\(^{2+}\) (mM) are given by the numbers on each curve. Purified membranes (225 \(\mu\)g of protein) were used; F\(^{-}\) absent.

![Graph 1](image1)

**Fig. 5.** Effect of F\(^{-}\) on skeletal muscle adenylate cyclase. A, effect of F\(^{-}\) concentrations on the reaction at 27\(^\circ\) and 37\(^\circ\). \([U-\sp{14}C]ATP\) (0.3 mM) was the substrate; Mg\(^{2+}\) was 6 mM; 95 \(\mu\)g of membrane protein were used. B, effect of temperature (12, 16, 23, 30, 38, and 43\(^\circ\)) on enzyme activity in the absence (○—○) and presence (Δ—Δ) of 12 mM F\(^{-}\). The substrate and Mg\(^{2+}\) concentrations were the same as in A; 183 \(\mu\)g of membrane protein were used.

3 to 5 mM; Mn\(^{2+}\) and Ca\(^{2+}\) stimulated at lower concentrations (\(K_m\), 1 to 2 mM) and became inhibitory at concentrations above 5 mM. Calcium ion inhibited the enzyme; when assayed in the presence of 18 mM Mg\(^{2+}\), half-maximal inhibition of basal or epinephrine-stimulated activity was produced by 0.5 mM Ca\(^{2+}\). The effect of three fixed concentrations of Mg\(^{2+}\) (3, 6, and 15 mM) on ATP saturation of the enzyme is shown in Fig. 4. Increasing the Mg\(^{2+}\) concentration increased the reaction velocity at all ATP concentrations. The \(K_m\) for ATP was estimated to be approximately 0.3 mM when Mg\(^{2+}\) was present in excess. At higher concentrations ATP profoundly inhibited enzyme activity (Fig. 4) and this inhibition was overcome by increasing the Mg\(^{2+}\) concentration.

**Stimulation by Flouoride**—Fluoride stimulates adenylate cyclase from most mammalian sources. The skeletal muscle membrane enzyme was stimulated 10- to 20-fold by this anion. The effect of F\(^{-}\) on the enzyme at two temperatures (27\(^\circ\) and 37\(^\circ\)) is shown in Fig. 5A. Maximal activity was achieved at 12 mM; half-maximal stimulation occurred at 4 mM. The effect of temperature from 12-43\(^\circ\) on basal and F\(^{-}\)-stimulated enzyme activity is presented in Fig. 5B in the form of an Arrhenius plot. There is a linear relationship between activity and temperature, except at 43\(^\circ\), where presumably inactivation occurs. Energies of activation calculated from the slopes are 7.8 kcal per mole for basal activity and 17.4 kcal per mole for F\(^{-}\)-stimulated activity.

**Fluoride Stimulation of Adenylate Cyclase** from brain (28), parotid gland (29), and adrenal (30, 31) has been shown to be irreversible. To examine this possibility in skeletal muscle, membrane preparations were incubated with Mg\(^{2+}\), with F\(^{-}\), and with Mg\(^{2+}\) plus F\(^{-}\), for 30 min at 4\(^\circ\). Following dialysis, samples were assayed in the presence of 9 mM Mg\(^{2+}\) (Table VII). Dialysis of control preparations (previously incubated with no additions) showed that the enzyme did not lose activity during this procedure. Preparations previously incubated with Mg\(^{2+}\), followed by dialysis, were totally inactive unless this cation was present in the assay, indicating the complete reversibility of Mg\(^{2+}\) stimulation. Membrane samples that had been previously incubated with F\(^{-}\) or F\(^{-}\) plus Mg\(^{2+}\), showed an 8-fold stimulation over basal activity when assayed after dialysis in the absence of F\(^{-}\). This corresponded to about 40% of the F\(^{-}\)-stimulated activity of the dialyzed control. Analysis for F\(^{-}\) by the Amadac-F reagent (which can detect as little as 5 to 10 \(\mu\)g of F\(^{-}\)) indicated F\(^{-}\) had been completely removed by dialysis. Identical results were obtained when the prior incubations were carried out for 10 min at 25\(^\circ\). The results suggest that F\(^{-}\) stimulation of the skeletal muscle enzyme is at least partially irreversible.

**Stimulation by Hormones**—Stimulation of adenylate cyclase by epinephrine in dog skeletal homogenates was reported by Klainer et al. (32) in 1962. Catecholamines stimulated adenylate cyclase activity in skeletal muscle membranes 2- to 4-fold. Dose-response curves for isopropylnorepinephrine, epinephrine, and noradrenalin are shown in Fig. 6. Concentrations required

| Prior incubation additions | Assay addition (F\(^{-}\), 12 mM) | Enzyme activity |
|---------------------------|---------------------------------|-----------------|
| A. Before dialysis        | None                            | --              |
| B. After dialysis         | None                            | 28              |
|                           | None                            | 253             |
|                           | Mg\(^{2+}\) (9 mM) plus F\(^{-}\) | 245             |
|                           | F\(^{-}\) (12 mM)                | --              |

From Table VII, it is evident that the irreversible stimulation of adenylate cyclase by fluoride is due to the relatively high concentration (12 mM) used in the assay. When assays were performed at lower substrate concentrations, the enzyme was completely reversible even when incubated with Mg\(^{2+}\) plus F\(^{-}\). The reason for this difference is not known, but it is possible that the enzyme might be reversibly inhibited at these concentrations, or that the irreversible stimulation might be due to fluoride inhibition of another enzyme in the assay system.
for half-maximal stimulation were 0.15, 0.5, and 10 μM, respectively. Phenylephrine (0.1 mM) stimulated activity 2.1-fold under conditions in which epinephrine at the same concentration stimulated 3.4-fold. Ephedrine (0.1 mM) was inactive. Stimulation due to epinephrine was blocked by propranolol (0.1 mM); propranolol had no effect on basal activity. Carbachol (0.1 mM) and several prostaglandins (E₁, E₃, F₁₂, F₂₅, and A₂) at 0.01 mM did not affect basal activity. Preliminary incubation of the enzyme in reaction mixtures lacking only substrate for various times (10 to 30 min) with insulin in concentrations varying from 1 milliunit per ml to 10 units per ml did not alter either basal or epinephrine-stimulated activity.

Nature of Neurohormone and F⁻ Stimulation—In our studies with the cardiac enzyme (6, 7), it was shown that the prominent action of F⁻ and epinephrine was to increase V₅₀ of the Mg²⁺-bound enzyme. The action of F⁻ (4 and 12 mM) and epinephrine (0.1 mM) on Mg²⁺ saturation of the skeletal muscle enzyme is shown in Fig. 7. Both F⁻ (Fig. 7A) and epinephrine (B) increased reaction velocity at all Mg²⁺ concentrations. The predominant kinetic effect was to increase V₅₀; there was no appreciable effect on the Kₘ for Mg²⁺. The effect of F⁻ (12 mM) and epinephrine (0.01 mM) on ATP saturation in the presence of two fixed Mg²⁺ concentrations (2.0 and 9.0 mM) is seen in Fig. 8. Under these conditions, both F⁻ (A) and epinephrine (B) stimulated the enzyme at all ATP concentrations. As previously observed (Fig. 4), increasing ATP concentrations were inhibitory. This inhibition was not reversed by F⁻ or epinephrine; it was reversed by increasing the Mg²⁺ concentration to 9 mM. The action of both F⁻ and epinephrine was to increase V₅₀. The affinity of the enzyme for ATP was not altered substantially by these agents. This is most clearly seen with 9 mM Mg²⁺ where ATP was not inhibitory. We conclude from these experiments that the kinetic nature of F⁻ and epinephrine stimulation of the skeletal muscle enzyme is essentially identical with that of the cardiac enzyme (6, 7).

Effect of Pyrophosphate—Pyrophosphate is a product of the adenylate cyclase reaction (33, 34). Addition of pyrophosphate at concentrations of 0.1 to 2 mM to the assay resulted in inhibition of basal, epinephrine-stimulated, and F⁻-stimulated adenylate cyclase activity (Fig. 9). The F⁻-stimulated activity was much more sensitive to pyrophosphate inhibition than basal or epinephrine-stimulated activities, and was observed at F⁻ concentrations varying from 2 to 10 mM. Pyrophosphate (2 mM) has been shown to inhibit adenylate cyclase from Escherichia coli (35), and a differential response to this agent was observed for glucagon and F⁻-stimulated adenylate cyclase in plasma membranes from liver (36). The effect of two fixed concentrations of pyrophos-
FIG. 9. Action of pyrophosphate on skeletal muscle adenylate cyclase. The assay contained 0.5 mM [U-32P]ATP, 6 mM Mg2+, and 350 μg of membrane protein. Activity was measured in the presence of varying concentrations of pyrophosphate as indicated, with no additions (○—○), with 0.01 mM epinephrine (Δ—Δ), or 12 mM F− (□—□).

FIG. 10. Effect of pyrophosphate on adenylate cyclase with varying ATP concentration. Membrane protein (290 μg) was assayed with increasing [γ-32P]ATP in the presence of fixed concentrations of pyrophosphate (concentration, mM, indicated by the numbers on each curve). Concentration of Mg2+ was 9 mM; of F−, 12 mM.

has also found the enzyme present in particulate fractions sedimenting at 18,800 × g. The difference between these results and ours may arise from the longer periods of homogenization employed (3, 44) which could result in disruption of the sarcoplasmic membrane, yielding particulate fragments sedimenting at higher gravitational forces. Our results indicate that a considerable portion of adenylate cyclase in skeletal muscle resides in the plasma membrane. A number of methods exist for the preparation of skeletal muscle sarclemma (4, 5, 23, 24, 45, 46). The procedure we have employed is a modification of the method of Kono and Colowick (4) and yields plasma membranes with an excellent yield of adenylate cyclase in a relatively short time interval. This is important because of the lability of the enzyme to storage even at 4°C. A number of criteria were employed to examine the purity of the membrane preparation. Phase contrast microscopy revealed empty, transparent, saclike structures similar to those previously reported (4, 5, 45, 46). The lipid composition was similar to that reported by Fiehn et al. (27), and the high molar ratio of cholesterol to phospholipid is characteristic of plasma membranes (28). The absence of cytochrome c oxidase, acid maltase, acid phosphatase, and pyrophosphatase activities indicates little or no contamination by mitochondria or lysosomes. The presence of Mg2+-ATPase, Na+K+-stimulated Mg2+-ATPase, and Ca2+-ATPase (Table IV) is also consis-

Discussion

Adenylate cyclase has been shown to be present in plasma membranes prepared from erythrocytes (37, 38), fat cells (39, 40), liver (41), and thyroid tissue (42, 43). In skeletal muscle, the enzyme has been previously reported to be present primarily in the mitochondrial and microsomal fractions (3). Hofman (44)
be further stimulated either by Na⁺ or K⁺ (but not as Na⁺ + K⁺-stimulated) and that such stimulation is insensitive to ouabain; the activity in the membrane preparations described here is inhibited by the glycoside (Table IV).

The skeletal muscle preparation possessed the ability to bind calcium ions in an ATP-dependent manner; binding was further enhanced by the addition of phosphate (Table V). Calcium has also been reported to bind to plasma membranes from bullfrog skeletal muscle (50). Microsomal contamination is not likely to account for our results since although heavy microsomes were present in the first 2000 x g residue, they were effectively removed by the series of washes and low speed centrifugation steps employed. In addition, extraction of sarcoplasmic reticulum fragments with 0.4 M LiBr under identical conditions greatly reduced their ability to sequester calcium. This has also been observed for cardiac sarcoplasmic reticulum (51). Several investigators have failed to observe energy-linked calcium binding to skeletal muscle membrane fractions. This could possibly be due to lability of the calcium-binding mechanism, to high salt concentrations, and to long periods of extraction. Recently we have observed that sarcoldemonal membranes prepared by the method of McCollister (5) bound very small amounts of calcium, whereas those prepared by a modification of the method of Rosenthal, Edelman, and Schwartz (45) bound this cation in amounts comparable to our preparation. Calcium binding to other cell plasma membranes, particularly liver (52) and erythrocyte ghosts (53, 54), has been observed. Perhaps this is a general property of cell membranes. All of the criteria cited above lead us to conclude that the membrane fraction consists of plasma membranes in a high degree of purity. Because of the excellent yield of adenylate cyclase and the ease and speed of isolation, we feel this preparation offers considerable promise for examination of hormonal binding and for further purification and especially solubilization studies.

The kinetic properties of adenylate cyclase in skeletal muscle are qualitatively similar to those previously reported for the myocardial enzyme (6, 7). The $K_a$ for ATP was about 0.3 mM; increasing concentrations of ATP were inhibitory. ATP inhibition was reversed by Mg²⁺; making it likely that ATP inhibition results from a competition between enzyme and nucleotide for free metal ions as has been proposed for the cardiac enzyme (7). Magnesium seems able to bind to the enzyme in addition to that involved at the catalytic site since enzyme activity was increased by Mg²⁺ concentrations greatly in excess of ATP (Figs. 3 and 4). The $K_a$ for Mg²⁺ was 2 to 5 mM. The consequence of Mg²⁺ binding to an apparent second site was enhanced reactivity of the catalytic site for substrate. Fluoride profoundly stimulated skeletal muscle adenylate cyclase. Stimulation occurred at all Mg²⁺ concentrations (Fig. 4A). The $K_a$ for Mg²⁺ was not appreciably altered by the union, nor was the affinity for substrate. Fluoride did not reverse ATP inhibition (Fig. 8).

In agreement with other investigators (28-31), stimulation due to F⁻ was partially irreversible (Table VII). Temperature had no effect on either basal or epinephrine-stimulated adenylate cyclase activity. These results are in accord with the findings of Craig et al. (56) that the activation of muscle glyogen synthetase by insulin was not associated with changes in tissue levels of cyclic AMP. The kinetic nature of epinephrine stimulation was similar to that of F⁻. Epinephrine increased reaction velocity at all Mg²⁺ and ATP concentrations (Figs. 7B and 8). The $K_a$ for Mg²⁺ and the $K_a$ for substrate were not appreciably altered; the primary action of epinephrine was to increase $V_{max}$, i.e. to increase catalytic reactivity. Increased reaction velocity at all Mg²⁺ and ATP concentrations has also been observed for glucagon and F⁻ on liver plasma membranes (41) and for thyroid-stimulating hormone and F⁻ on thyroid plasma membranes (43). Adenylate cyclase in skeletal muscle may exist as a free enzyme or in a magnesium-bound form, both forms having equal affinity for substrate, with the magnesium-bound enzyme having greater catalytic reactivity. Similar alterations in conformation may occur from interaction with F⁻ or epinephrine with resultant increased maximal velocity without alterations in the affinity for Mg²⁺ or for substrate. Thus the regulation of adenylate cyclase in skeletal muscle seems in accord with that of a "V" allosteric enzyme, using the terminology of Monod, Wyman, and Changeux (57). The same mechanism for regulation has been applied to protein kinase from skeletal muscle (58).

Results obtained on hormonal regulation derived from in vitro studies of adenylate cyclase must be interpreted with caution. For example, responses to catecholamines require concentrations much higher than those which are effective physiologically and the skeletal muscle membrane preparation (Fig. 6) is no exception. In this regard, it is pertinent to refer to the findings of Stull and Mayer (50) in which the formation of phosphorylase $a$ in rabbit gracilis muscle in response to low doses of isoproterenol-norepinephrine was dissociated from increases in cyclic AMP levels and the activation of phosphorylase $b$ kinase. Higher doses of the catecholamine resulted in phosphorylase activation which was correlated with increased levels of cyclic AMP. The examination of adenylate cyclase regulation in membrane preparations may, therefore, not provide final definitive answers concerning regulatory mechanisms in intact cells and tissues. The present studies also suggest that adenylate cyclase activity is higher in red skeletal muscle fibers than in white fibers. The former are equipped for oxidative metabolism and the oxidation of lipids. In accord with this, it has been found that exercise-induced lipolysis required normal hormonal mechanisms, whereas exercise-induced glycogenolysis was not influenced by adrenalectomy or adrenergic blockade (80), i.e. glycogenolysis occurred in animals devoid of hormonal regulation. Our understanding of the role of the adenylate cyclase system in regulating energy supply to tissues, although expanding rapidly, is not yet complete.
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