β-Adrenergic Receptor and Adenylate Cyclase in Transverse Tubules of Skeletal Muscle*

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Recent work on isolated cardiac and skeletal muscle organelles has raised certain questions concerning the details of β-adrenergic action in muscle. Since the original description of stimulation of calcium accumulation in microsomes by adenosine 3',5'-monophosphate, several authors have described a similar effect caused by epinephrine (1–5). Two explanations may account for this: 1) the hormone receptor and adenylate cyclase are located in the sarcoplasmic reticulum membrane; 2) the epinephrine sensitivity and adenylate cyclase activity are associated with external membrane which contaminates the cardiac microsome preparation. The first explanation requires a radical modification of the basic scheme of adrenergic influence on cardiac muscle. Entman et al. (6) have presented evidence for the presence of β-adrenergic receptor and adenylate cyclase in sarcoplasmic reticulum of both cardiac and skeletal muscle and have suggested the existence of an internal β-adrenergic receptor.

The transmission of the hormonal message through cAMP to fast skeletal muscle microsomes to increase calcium accumulation by these organelles has also been the subject of controversy. The effects on contractility of β-adrenergic agents in fast skeletal muscle are very much lower than those in heart and have been postulated to be mediated purely by local vasoconstriction (7). On the other hand, epinephrine is known to induce glycogenolysis (8). In support of a direct effect of epinephrine on contraction, some recent work has shown that cAMP and protein kinase caused enhanced calcium accumulation by microsomes of fast muscle of cat (9) and rabbit (10), although Kirchberger and Tada (11) were unable to observe any effect of cAMP and protein kinase on calcium movement in fast skeletal muscle microsomes from rabbit.

The work described in this paper was initiated in order to clarify some of the problems raised by the experimental observations described above. We have recently been successful in fractionating fast skeletal muscle microsomes into three distinct fractions. These are longitudinal reticulum, terminal cisternae, and transverse tubules (12, 13).

In this paper, we describe our findings on the subcellular location of microsomal β-adrenergic receptor and hormone-stimulated adenylate cyclase and investigate the phosphorylation of microsomal fractions stimulated by protein kinase and cAMP.

MATERIALS AND METHODS

Preparation of Organelles—Sarcopinissalis muscle of 3½-lb rabbits was employed. The preparation of microsomes, density gradient centrifugation, and isolation of transverse tubules were carried out as described previously (12, 13). A summary of this is as follows.

Skeletal muscle microsomes prepared by conventional techniques were layered on a sucrose density gradient. They formed two distinct bands. The "light band" at 30% sucrose consists mainly of sarcoplasmic reticulum containing electron dense matter and of similar vesicles with attached T-tubules in the form of intact triad junctions (19). Electron microscopic evidence supports the view that this band is mainly of T-tubules, transverse tubules; Hepes, 4-(2-hydroxyethyl)piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetracetic acid; Gpp(NH)p, guanyl-5'-yl imidodiphosphate.

1 The abbreviations used are: cAMP, adenosine 3',5'-monophosphate; T-tubules, transverse tubules; Hepes, 4-(2-hydroxyethyl)piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetracetic acid; Gpp(NH)p, guanyl-5'-yl imidodiphosphate.
terminal cisternae origin (12, 14). The T-tubules were detached from the terminal cisternae by passing the suspension through a French press and were separated from the terminal cisternae as a distinct band in a sucrose density gradient. We have also devised a technique for tracking T-tubules. The intact muscle was injected with \[^{3}H\]ouabain. The ouabain becomes bound and trapped in the T-tubules and remains associated with them through the whole fractionation process (12, 13).

In these experiments, in the isolation of T-tubules, a KCl wash was sometimes employed in order to obtain a slightly improved separation of T-tubules from terminal cisternae. When this was used, the heavy band from the microsomal gradient was diluted with 2 volumes of 0.9 \(\mu\)M KCl and centrifuged at 96,000 \(\times\) g for 70 min. The pellet was resuspended in 0.25 \(\mu\)l sucrose for passage through the French press. Otherwise, the procedure was identical with that described.

**Assays of Binding of (-)-\[^{3}H\]dihydroalprenolol**

To determine the number of \(\beta\)-adrenergic receptors in skeletal muscle, the specific binding of (-)-\[^{3}H\]dihydroalprenolol to a preparation of terminal cisternae was measured. Specific binding was defined as the difference between the binding in the presence and absence of 10 \(\mu\)M (-)-alprenolol. The results are expressed as the number of receptors per milligram of protein.

The control assay medium for dihydroalprenolol consisted of 40 \(\mu\)M histidine, pH 6.8, 130 \(\mu\)M KCl, 100 \(\mu\)M sodium EGTA, 2 \(\mu\)M MgCl\(_2\), 0.5 \(\mu\)M ATP containing 1 \(\mu\)Ci \[^{32}P\]ATP in a volume of 0.2 ml. The test medium consisted of 8.0, 4.0, 10 mM MgCl\(_2\), and filtered under vacuum through a Whatman GF/C glass fiber filter. Each filter was then washed with a further 12 ml of ice cold buffer, the total time between dilution and the end of the wash being less than 15 s. The filters were placed in counting vials, dried in an oven at about 40°C for an hour, and counted with 5 ml of a Triton X-100/toluene-base scintillation fluid. The counting efficiency in the scintillation counter was found to be 35%.

**Adenylate Cyclase Assays**

The incubation medium contained 1.6 mM Tris-HCl (pH 7.4) and 0.2 mM \[^{32}P\]ATP (20 cpm/pmol), 5 mM MgCl\(_2\), 30 mM KCl, 0.5 mM EGTA, 10 mM theophylline, 0.01% bovine serum albumin, 40 mM Tris-Cl, pH 7.6, in a final volume of 0.1 ml. \[^{3}H\]AMP (5000 cpm/mg) present in the incubation medium as an internal control to correct for losses of cAMP. Separation of the labeled product from substrate was achieved by the method of Ramachandran (16), using a column of dry neutral alumina.

For the measurement of the cyclase activity in fractions from sucrose gradients, 8 \(\mu\)M NaF was present. Reactions were started by the addition of 25 \(\mu\)l of the individual fractions, incubated for 10 min, and stopped by the addition of 0.1% Triton X-100, 10 mM EDTA. For these assays, a regenerating system was not used since the ATP-regenerating system was not found to be necessary. ATP, 2.5 mM THF, 4 mM MgCl\(_2\), 0.2 mM Tris-EDTA, pH 7.4. Microsomes were prepared in a manner identical with that employed for skeletal muscle and the preparation was centrifuged in a continuous sucrose density gradient to form two distinct bands. The lower band at approximately 1100 \(\mu\)g protein was diluted with 2 volumes of 0.9 \(\mu\)M KCl and centrifuged for 70 min at 80,000 \(\times\) g. The pellet was resuspended in 2 ml of 350 \(\mu\)M ouabain and assayed for protein kinase-stimulated phosphorylation as described below; 1 mg/ml of membrane protein was employed in the assay. Self-phosphorylation (in the absence of ATP) of protein kinase and microsomal phosphorylation in the absence of protein kinase and cAMP were subtracted as controls. Phosphorylation of cardiac microsomes was found to be 0.18 nmol/mg of protein in 10 min. The degree of phosphorylation was not altered by inclusion of 10 \(\mu\)M NaF and 2.5 mM theophylline in the assay medium.

**RESULTS**

**Adrennergic Receptor**—In Figs. 1 and 2 we establish the utility of \[^{3}H\]dihydroalprenolol as a selective ligand to detect and assay the \(\beta\)-adrenergic receptor in skeletal muscle as has been described for other tissues (18). The binding properties of (-)-\[^{3}H\]dihydroalprenolol to a preparation of terminal cisternae with attached T-tubules are shown in Fig. 1A. The nonspecific binding component in the presence of (-)-alprenolol appears as a linear function of (-)-\[^{3}H\]dihydroalprenolol (open circles). Specific (\(-\)^3H)dihydroalprenolol binding appears as a hyperbolic function, and a Scatchard plot of these data (Fig. 1B) shows within the limits of accuracy of the assay a single binding site with \(K_d\) of 2.8 nM and a density of binding sites of 0.194 pmol/mg of membrane protein.

**Fig. 2** shows the effects of several \(\beta\)-adrenergic agonists in inhibiting the binding of \(-\)\[^{3}H\]dihydroalprenolol. The most potent inhibitor of binding of \(-\)\[^{3}H\]dihydroalprenolol is (-)-isoproterenol which gives a half-maximal inhibition at 2 \(\times\) 10\(^{-7}\) M in the presence of 5 nM (-)-\[^{3}H\]dihydroalprenolol. The stereospecificity of the inhibition is demonstrated by the much higher concentration of (+)-isoproterenol required for half-maximal inhibition of \(-\)\[^{3}H\]dihydroalprenolol.
β-Adrenergic Receptor in Skeletal Muscle

FIG. 1. Specific and nonspecific binding of dihydroalprenolol to fractionated skeletal muscle microsomes. The incubation conditions of (-)-[3H)dihydroalprenolol (DHA) with membranes have been described under "Materials and Methods." In each assay, 1.2 mg/ml of "heavy band" microsomes were employed. Specific binding is shown by closed circles. The free (-)-[3H]dihydroalprenolol was calculated by subtracting the bound (-)-[3H]dihydroalprenolol. The nonspecific binding (open circles) was that obtained in the presence of 10 nM (-)-alprenolol. A shows linear plots of binding while B is a Scatchard plot of the specifically bound (-)-[3H]dihydroalprenolol.

FIG. 2. Inhibition of (-)-[3H]dihydroalprenolol binding by adrenergic agonists. Conditions of incubation are as described under "Materials and Methods" in which 1.2 mg/ml of protein from the heavy microsomal band was present. Inhibition curves are: O--O, (-)-isoproterenol; O-.-O, (-)-epinephrine; n -—— n, (+)-isoproterenol; 0. t 0, (-)-norepinephrine. The potency of the (+)-isomer is approximately 2 orders of magnitude lower than that of the (-)-isomer. The relative potency of adrenergic agonists is in the order isoproterenol > epinephrine > norepinephrine. This potency series is consistent with the potency ratios for a β-adrenergic response. The slope of the inhibition curves for the adrenergic agonists is low (Hill coefficient = 0.7) as has also been observed in other organs (18, 19).

Fig. 3 shows the (-)-[3H]dihydroalprenolol binding profiles of microsomes which have been centrifuged on a continuous sucrose density gradient. The protein profile shows two bands at isopycnic points of 30 and 39% sucrose. We have shown previously that the light band is mainly longitudinal reticulum, while the heavy band contains terminal cisternae and attached T-tubules (12). (-)-[3H]dihydroalprenolol binding activity in each fraction from the gradient is shown by the solid line of the lower curve. This appears as a single band with a peak at 39% sucrose. The position and distribution of (-)-[3H]dihydroalprenolol binding conforms almost exactly with that of the heavy band in the protein profile. No discernible (-)-[3H]dihydroalprenolol band is present in the longitudinal reticulum region. The dotted line indicates the specific activity of (-)-[3H]dihydroalprenolol binding. In the region of the terminal cisterna band from 44 to 35% sucrose, the specific activity is constant within experimental error. This supports the view that the binding activity is an intrinsic property of the band rather than a reflection of the presence of contaminating vesicles. The density of binding sites is 0.15 pmol/mg of protein. According to Fig. 1B, the concentration of (-)-[3H]dihydroalprenolol employed should give approximately 65% saturation of the high affinity binding sites, hence the density of β-adrenergic receptors in this band is approximately 0.23 pmol/mg of protein.

Since the heavy band from the microsomal gradient contains both terminal cisternae and T-tubules equally distributed in this gradient, we carried out further fractionalization to separate these organelles. The intact muscle was injected with [3H]ouabain to label the T-tubules. The heavy band from the first gradient was washed with KCl, passed through a French press, and layered on a sucrose density gradient. Fig. 4 shows the protein, (-)-[3H]dihydroalprenolol, and ouabain profiles from this gradient. The protein profile shows bands at 32 and 38% sucrose which correspond to fragmented terminal cisternae. A shoulder at 20% sucrose delineates the T-tubular region (13). However, the distribution of T-tubules in this gradient is more accurately reflected by the [3H]ouabain profile, which shows a single band at 20% sucrose. The
(-)-[3H]dihydroalprenolol binding activity is concentrated in a single band at 20% sucrose. The distribution of this band is in almost perfect conformity with the [3H]ouabain distribution. Thus, (-)-[3H]dihydroalprenolol binding is concentrated in the T-tubule band with no detectable activity in the terminal cisterna region of the gradient. The specific activity profile (dashed trace) shows a maximum of 0.4 pmol/mg of membrane protein which corresponds to a density of receptors of 0.61 pmol/mg of protein in the T-tubular band.

Adenylate Cyclase - The adenylate cyclase activity and hormone sensitivity of a preparation of terminal cisternae with attached T-tubules are shown in Table I. Isoproterenol stimulates the cyclase by a factor of 2.9. NaF and the GTP analog, Gpp(NH)p, also activate the cyclase. There is a notable further stimulation of the cyclase activity by a factor of 2.9 in the presence of Gpp(NH)p when isoproterenol is added.

Fig. 5 shows the adenylate cyclase activity and protein profile of microsomes separated in a density gradient. The protein profile is basically identical with that of Fig. 3. The adenylate cyclase profile shows a single band with peak at 38% sucrose coincident with the terminal cisterna-triad band. No significant activity is present in the longitudinal reticulum band. The specific activity of adenylate cyclase in the heavy band is approximately 60 pmol/mg of membrane protein/min.

Fig. 6 shows the protein profile, adenylate cyclase, and

![Graph 1](image1)

![Graph 2](image2)

![Graph 3](image3)

**Table I**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Adenylate cyclase pmol/mg protein-min ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>13 ± 7</td>
</tr>
<tr>
<td>10^-5 M Isoproterenol</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>10^-4 M Isoproterenol</td>
<td>39 ± 7</td>
</tr>
<tr>
<td>4 mM NaF</td>
<td>58 ± 5</td>
</tr>
<tr>
<td>8 mM NaF</td>
<td>111 ± 4</td>
</tr>
<tr>
<td>10^-4 M Gpp(NH)p</td>
<td>78 ± 14</td>
</tr>
<tr>
<td>10^-4 M Gpp(NH)p + 10^-4 M isoproterenol</td>
<td>170 ± 8</td>
</tr>
<tr>
<td>10^-4 M Gpp(NH)p + 8 mM NaF</td>
<td>122 ± 30</td>
</tr>
</tbody>
</table>

* S.E. estimates are based on triplicate assays.

![Graph 4](image4)

**Fig. 4.** Protein profile, (-)-[3H]dihydroalprenolol, and ouabain binding from a sucrose gradient of the heavy microsomal band after passage through a French press. A KCl wash was employed in preparing the gradient as described under "Materials and Methods." After the animal was killed, the intact muscle was injected with 10 μCi of [3H]ouabain in a Krebs-Ringers solution described previously (12) and incubated for 30 min before the muscle was excised. The conditions for (-)-[3H]dihydroalprenolol binding are those of Fig. 3. Specific activity is shown by open circles.

**Fig. 5.** Protein profile and adenylate cyclase activity from a sucrose gradient of microsomes. Adenylate cyclase was assayed as described under "Materials and Methods" in the presence of 8 mM NaF. Specific activity is shown by open circles.

**Fig. 6.** Protein profile, adenylate cyclase, and ouabain binding from a sucrose gradient of the heavy microsomal band after passage through a French press. Conditions of organelle preparation are the same as those for Fig. 4 except that a KCl wash was not employed. Specific activity is shown by open circles.
[\text{H}]tubulin activities after separation of terminal cisternae from T-tubules using a French press. The characteristic terminal cisternae bands are seen at 32 and 30% sucrose. The absence of a KCl wash in this preparation accounts for the different isopycnic points compared with those of Fig. 4. The protein shoulder and [\text{H}]tubulin band are located at 21% sucrose corresponding to T-tubules. We are unable to detect significant activity of adenylyl cyclase in the terminal cisterma region of the gradient. The activity is concentrated as a single band with isopycnic point at 21% sucrose, and shows a correspondence both of peak position and of distribution with the [\text{H}]tubulin activity. The specific activity of the F-stimulated adenylyl cyclase at the peak position is approximately 2.3 nmol/mg of membrane protein/min. We therefore conclude that the microsomal adenylyl cyclase is located in the T-tubules.

Protein Kinase-stimulated Phosphorylation — In a continuation of our determinations on the morphological sources of adrenergic control of fast skeletal muscle, we have assayed for phosphorylation of microsomes induced by cAMP and protein kinase. As a control for the activity of the preparation of skeletal muscle protein kinase, we assayed for phosphorylation of a preparation of heart muscle microsomes. The assay showed 0.18 nmol of phosphate incorporated from [\gamma-\text{P}]ATP/mg of protein in 10 min. This value is comparable to that obtained by Kirchberger et al. (1). However, when we assayed both the light band and heavy band of fast skeletal muscle microsomes, we were unable to detect any phosphorylation using identical assay conditions. Our methods would permit us to detect 0.02 nmol of phosphorylation/mg of protein in 10 min. This experiment was attempted with three separate preparations of microsomes without detecting any phosphorylation.

DISCUSSION

Our data show the presence of \(\beta\)-adrenergic receptors and of hormone-stimulated adenylyl cyclase in transverse tubules of skeletal muscle. The density of receptors and the adenylyl cyclase in the T-tubules are fully comparable to those found in isolated membranes from other organs which exhibit \(\beta\)-adrenergic activity (20-22). We were unable to detect significant amounts of either protein in longitudinal reticulum or terminal cisternae. We infer that the presence of \(\beta\)-adrenergic receptor and of adenylyl cyclase activity in microsomes from skeletal or heart muscle does not require the postulate of an internal receptor system.

The criteria which we have employed to determine organelle location of these proteins have been to show a total correlation between organelles and protein activity on two types of density gradients. Thus, we have been able to demonstrate this correlation of activity with T-tubules, employing the four parameters of position and distribution on two gradients. We believe that this approach is more accurate than that of assaying for impurities in a batch preparation.

\(\beta\)-Adrenergic receptor and adenylyl cyclase may also be present in plasma membrane of skeletal muscle and we have no data concerning this. However, we have demonstrated previously that the ouabain binding vesicles found in microsomes which migrate with terminal cisternae in the first density gradient are T-tubules rather than plasma membrane (13). The correlation of \((-\text{[H]}\text{dihydroalprenolol binding and adenylyl cyclase activity in these microsomal density gradients is entirely with T-tubules. Thus, in the first gradient, the activity is found in exact correspondence with the heavy band which contains morphologically identified terminal cisternae and attached T-tubules. In the density gradient after French press treatment, we find an almost perfect coincidence of activity with the ouabain binding vesicles.

We have not succeeded in elucidating the role of \(\beta\)-adrenergic agents in indirectly influencing skeletal muscle sarcoplasmic reticulum. We were not able to detect phosphorylation of skeletal muscle microsomes by ATP, skeletal muscle protein kinase, and CAMP although in a similar assay of cardiac microsomes, we detected phosphorylation. In this, we confirm the similar conclusions of Kirchberger and Tada (11). However, Schwartz et al. (9) have presented evidence that fast skeletal muscle microsomes are phosphorylated by protein kinase. These authors appear to have argued that EGTA inhibits phosphorylation, although the only positive data presented were in experiments conducted in the presence of EGTA. In our unsuccessful attempt to confirm their data, we performed our assays in the presence of EGTA, but we have not investigated phosphorylation in the absence of EGTA.

The presence of \(\beta\)-adrenergic receptor and adenylyl cyclase in T-tubules is consistent with the classic mechanism of \(\beta\)-adrenergic action in which the receptor and cyclase transduce the message from extracellular epinephrine to cause increased levels of cytoplasmic cAMP. The effects of cAMP may then be mediated through cytoplasmic enzymes or enzymes in plasma membrane or internal organelles. The major known effects of catecholamines on skeletal muscle are from circulating epinephrine. The time delay for permeation of epinephrine into the deep invaginations of the T-tubules is unlikely to be significant in the context of the time for adrenal epinephrine to exert metabolic effects.

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