Activation of Phosphorylase in Frog Muscle as Determined by Contractile Activity

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ABSTRACT  The state of activation of phosphorylation in muscle has been reinvestigated by combining the extraction procedures of Danforth, Helmreich, and Cori with the low-temperature techniques of this laboratory. In resting frog muscle, the phosphorylase-a content is usually below detectability. Upon contractile activity in series of twitches, activation of phosphorylase b to a took place, without activation of phosphorylase b kinase as defined by the assay procedure. Two different experimental designs were used to examine the relation between phosphorylase activation and the myothermally determined energy turnover per twitch, and these showed, identically, that the enzyme activation is proportional to the energy per twitch.

As the result of investigations by C. F. and G. T. Cori and associates (1, 2, 3), it has become known that glycogen phosphorylase in muscle occurs in two forms a and b, and that activation or b-a transformation occurs upon contractile activity. Knowledge of the factors determining this activation process is of importance for two reasons. On the one hand, the process constitutes an example of a metabolic regulation, i.e. a mechanism for activation of metabolism upon the transition from rest to activity. In addition, however, it is known that the protein kinase involved can also act upon other proteins. While the functional meaning of such phosphorylations remains to be established, they may be of considerable importance. Thus, the activation of phosphorylase can also be seen as a precedent of mechanisms beyond metabolic regulation.

In this paper, we demonstrate that in resting frog muscle the amount of phosphorylase a is below detectability so that the enzyme can indeed be considered as a rate-limiting step for glycolysis in resting muscle. As to its activation after excitation we have shown that, under the chosen conditions of the experiments, its amount is determined by the energy turnover per twitch, indicating that the cell has means to assess its metabolic need also at this regulatory level.

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METHODS

Muscles and Their Treatment

All experiments were done on the semitendinosus muscle of *Rana pipiens*. These were dissected with the pelvic bone attached and handled as such throughout the experiment. Measured in the body with the legs extended perpendicularly, they were 30–33 mm long. Wet weights determined after the experiments were 60–70 mg per muscle. After dissection, the muscles were recovered by aeration in bicarbonate-Ringer in 95% O2–5% CO2 overnight at 3°C.

Muscle pairs were mounted in the current version of the immersion-freezing apparatus (4) and were kept in the chamber for 5 min at 0°C in 95% N2–5% CO2, unless described otherwise. They were then stimulated in that same situation with a series of 40 excitations. Stimulation was above threshold with 3.0-ms square pulses at 3.2-s intervals.

Muscles were varied as to the mechanical conditions of contraction as will be specified in the Results section. One set of treatments included rest and four levels of energy turnover. In another series, the energy utilization per cycle was varied in isometric twitches by extension beyond *L0* as in our previous work (5). The respective treatments within a series were varied at random over the individual experiments on approximately 20 muscles which made up a day’s program, apart from one special design which will be described separately. After the last contraction cycle, the muscles were frozen 200–500 ms after relaxation and kept at −80°C until biochemical assay.

Myothermal Measurements

These were performed to quantitate the energy turnover per twitch or per series under the selected conditions, and were done with the standard methods of this laboratory (5, 6).

Assay of Phosphorylase a and b

The muscles were extracted (see below) with methods designed to preserve the a content of phosphorylase at its in vivo level. The assays for phosphorylase a and b were the standard procedures based upon the original work by Cori et al. (7; Danforth et al., 8), and were begun within 20 min after the final centrifugation of the extracts. The assay medium is as follows: 0.2 ml of muscle extract 1:50; 0.2 ml of 0.001 M EDTA and 0.02 M NaF at pH 6.8; 0.2 ml of 4% purified glycogen; 0.2 ml of 0.3 M glucose-1-phosphate pH 6.8 for the determination of phosphorylase a, or of 0.3 M glucose-1-phosphate, and 0.002 M AMP pH 6.8 for the determination of total phosphorylase. The reaction mixture was incubated in a 30°C waterbath for 10 min, timed from the addition of the glucose-1-phosphate solution, after immersion for 3–5 min at the assay temperature. Aliquots of 0.2 ml were then diluted into 4.1 ml of 0.07 N H2SO4, and inorganic phosphate determined by the Fiske and Subbarow method. A blank value for each extract was determined by adding 0.15 ml of reaction into the acid and adding 0.05 ml glucose-1-phosphate, with and without AMP, afterwards. Analytical values are given as micromoles of inorganic phosphate per gram of muscle per minute.
Assay of Phosphorylase Kinase

This followed the procedures outlined by Krebs et al. (9) and Drummond et al. (10). The reaction mixture was made up as follows: 0.04 ml muscle extract 1:50; 0.04 ml of 0.05 M fresh neutralized cysteine; 0.16 ml of 0.125 M glycerophosphate buffer, of pH 6.8 or 8.2, respectively; 0.08 ml of phosphorylase b solution, 2 X crystallized, 50 mg per ml (AMP free, from Sigma Chemical Co., St. Louis, Mo.); 0.08 ml of neutral 0.018 M ATP, and 0.06 M Mg-acetate. The latter at 0.012 M is in vast excess over the 0.1 mM EDTA contributed by the extract.

Aliquots from each extract were run in the above mixtures at both pH 6.8 and 8.2; after preincubation at 30°C, the reaction was started by the addition of the ATP solution. After 5-min reaction time, 0.1-ml aliquots were transferred into 1.9 ml of 0.04 M glycerophosphate, 0.03 M cysteine, and 0.004 M EDTA at pH 6.8, which terminated the kinase reaction. Samples of 0.1 ml were then used to determine the phosphorylase a formed by adding to 0.1 ml of 0.04 M NaF pH 7.0, 0.1 ml of 4% glycogen and 0.1 ml of 0.3 M glucose-1-phosphate pH 6.8 and incubation for 10 min. Aliquots of 0.2 ml were used for phosphate, which was then determined as in the phosphorylase assays. Controls were the extracts treated identically but for the omission of phosphorylase b. Data were recorded in micromolar units of kinase activity, where one unit is catalyzing the formation of 100 μM units of phosphorylase a, which means that 100 μmol of inorganic phosphate is released per gram of muscle per minute. The activation of phosphorylase-b kinase is measured by the ratio of activities of pH 6.8 over 8.2.

RESULTS

Extraction and Fixation Technique for the Assessment of the Degree of Activation of Phosphorylase

This problem had been approached in the past by the Cori school by the inclusion of EDTA and NaF in the extraction medium, in order to block the action of the phosphorylase-activating and deactivating enzymes, respectively. When applying this procedure to muscles rapidly frozen and subsequently reduced in the frozen state to a very fine powder with our standard techniques, resting values of 20% a were obtained with considerable reproducibility (11). Danforth et al. (8) found resting values of the order of 1-5% by extracting at −35°C in the presence of 60% glycerol.

We have investigated the procedure by applying the extraction medium of Danforth et al. as follows. Extractant I consisted of 60% glycerol, 1 mM EDTA, 0.02 M NaF, 0.03 M glycerophosphate, and 0.03 M cysteine at pH 6.8. Extractant II was the same but without glycerol. The frozen muscles were powdered finely at liquid nitrogen temperature by shaking them with the cartridge and ball method developed by Seraydarian et al. (12). Extractant I was then added in a volume 10 times the muscle weight and the closed cartridge shaken for 40 s. This served an even mixing of powder and medium,
and a penetration of the muscle substance with inhibitors at \(-35^\circ C\) at which the kinase appears sufficiently inhibited. For effective extraction, 40 vol of extractant II was then added and the cartridge shaken for another 20 s, and immediately centrifuged at 0–3°C for 10 min.

This method had repeatedly shown (Tables I, II) that in resting muscle the phosphorylase \(a\) content was undetectable, and will be listed as zero. The significance of this finding will be set forth in the Discussion. For the moment, the practical aspect is that all increases due to contractile activity occur above a negligible background. The extraction method also served as the starting point for the assay of phosphorylase kinase.

**Total Amount of Phosphorylase**

The assays described for the determination of both phosphorylase \(a\) and \(b\) provide data for the estimation of the "maximal capacity" (17) of the total phosphorylase in our material. Some separate assays were done to assess the completeness of extraction, which showed that 0.89 ± 0.015 of the total phosphorylase were obtained in the first extraction and 0.11 ± 0.016 in the second one. Data are collected in Table II. This reveals an average catalytic capacity of 55 \(\mu\)mol/min/g muscle in the synthetic direction, or, by estimation (17, their Table VI) of 22 \(\mu\)mol min\(^{-1}\) g\(^{-1}\) in the glycogenolytic direction, at 30°C. For 20°C, to be used for certain comparisons, the figure can be set at 8–10 \(\mu\)mol min\(^{-1}\) g\(^{-1}\).

**Phosphorylase Activation under Different Conditions**

These were explored for the case of isometric contractions at standard length \(L_o\). This reference is somewhat less conveniently defined in the semitendinosus than in the sartorius muscle, but experiments devoted to that point showed a force maximum in the twitch at a length 3–4 mm (dependent on the size of the muscle) below the length \(L_{in situ}\) as defined above.

Table I shows the activation of phosphorylase achieved in series of different

#### Table I

| Number of | \(\times\) | Untreated (anaerobic) | Guanethidine (anaerobic) | IAA (anaerobic) | Aerobic |
|----------|-----------|-----------------------|-------------------------|----------------|---------|
|          | Ph        | K                     | Ph                      | Ph             | Ph      |
| 0        | 11        | 0.992 ± 0.022         | 0.116 ± 0.016           | 0.070 ± 0.015  |
| 10       | 0.080 ± 0.030 | 0.116 ± 0.016          | 0.090 ± 0.018           |
| 20       | 0.176 ± 0.048 | 0.115 ± 0.022          | 0.268 ± 0.039           |
| 40       | 0.367 ± 0.096 | 0.090 ± 0.016          | 0.201 ± 0.012           |
| 60       | 0.320 ± 0.036 | 0.115 ± 0.019          | 0.145 ± 0.021           |

Active phosphorylase is expressed in Ph columns as the fraction in the form of phosphorylase \(a\) and the active phosphorylase kinase is expressed in the K columns as the ratio of activities of pH 6.8 and 8.2.
twitch numbers at $L_o$. There is a linear increase up to 40 twitches, therefore, this number was selected for the later experiments. The question of the possible establishment of a steady-state level after this interval was not investigated.

In those experiments, and all others to be described subsequently, the muscles were held anaerobically, and not otherwise modified. Table I also shows that the phosphorylase activation was less both in iodoacetate-poisoned muscles and oxygenated muscles. It is not known what aspects of the activation are affected by iodoacetic acid (IAA), but clearly the drug causes no total inhibition, and indeed formation of intermediates up to the triosephosphate level is known to occur (13, 14).

**State of Activation of Phosphorylase Kinase**

The tables also list the results of phosphorylase kinase assays. The ratio of kinase activity at pH 6.8 to that at pH 8.2 (9) was about 0.11 under most circumstances, and did not change during the activity patterns that caused activation of phosphorylase $b$ and $a$. Therefore, the latter activation is effected by modulating the activity of the active kinase present under the circumstances.

Some efforts were made to modify the kinase by preincubating the muscles for 1 h at 0°C with 0.05 mM guanethidine added to the bicarbonate-Ringer medium. This caused a reduction of the ratio of kinase activity at pH 6.8:pH 8.2 by about half, suggesting some chronic influence of an adrenergic mechanism in the regulation of the resting kinase levels. After this treatment (Table I) there was some reduction of the phosphorylase activation in a standard series of isometric twitches, but the limited variation obtainable did not encourage further studies with the drug at this time, and it did not obviously reduce the standard error.

**Variation of Working Conditions**

We next turned to the study of the effect of varying the energy turnover per twitch (in 40-twitch series) upon phosphorylase activation. Energy turnover was varied by modes of activity of somewhat different functional connotations. Besides unstimulated controls, four different working conditions were selected: (i) maximally stretched muscles in which, due to the segregation of A- and I-filaments, no mechanical response occurs, and the only energy usage taking place is that linked with the excitation-contraction coupling cycle for which a corresponding amount of biochemical change has been shown (5), (ii) lightly loaded maximally shortening muscles in which it is necessary to restore the starting length $L_o$ by forced extension after each cycle is over, and in which the energy turnover, in addition to that under i, is essentially that associated with the shortening heat (6), (iii) isometrically contracting
muscles at standard length $L_0$, and (iv) muscles afterloaded at $L_0$ so as to give about maximal isotonic work. In each series, a number of myothermal measurements on randomly selected muscles were performed to determine the total energy in the selected modes of activity. It was hoped, on the basis of results in a preliminary series that these modes would correspond to energy turnover levels of the orders of (i) 0.5–1.0, (ii) 2, (iii) 3, and (iv) 3.5–4 mcal per cycle per gram. However, this spread of values cannot be controlled or predicted for a given population of animals, and the categories ii–iv are not always well separated.

In a preliminary series, a linear relation between energy turnover and phosphorylase activation was observed. However, the assays were still done with the technique of Guillory and Mommaerts (11) and consequently the variations were superimposed upon a considerable base line. It was after this experience that the new analytical technique was developed and also applied to phosphorylase-b kinase as well. A full series so done suffered from the circumstance that the modes ii, iii, and iv were clustered closely. This was particularly due to the circumstance that in mode iv one often falls short of optimal work performance if the loading is arbitrarily chosen. Therefore, in the final series of experiments, described here in full, special precautions were taken to assure best execution of mode iv. This was done by using two muscles of a pair for isometric (iii) and optimally loaded (iv) contraction. The former was then given eight test twitches to select the length setting for maximal isometric force $P_o$, whereas the latter was then set at that same length and afterloaded to 0.4 $P_o$; the muscles then received 32 additional and 50 stimuli, respectively. These particular results for iii designated as $(8+32)$, due to some differences in timing and in tension developed, were not used for the calculation of the regressions, though their results, entered in Fig. 1 and Table II, did fit well with the others. The results of this series are collected and evaluated in Table II. The following features emerge.

A considerable range of phosphorylase activations is encountered, but always without involving an increase of active phosphorylase kinase as defined. These phosphorylase-a contents are superimposed upon a level in the resting muscles which was below detectability, and is listed as zero, in all individual experiments.

When entered against the energy turnover per twitch, the data fit well to a linear regression relation. There is no indication of a finite intercept at zero energy per twitch, and the regressions are indistinguishable whether the resting control values are included or not (Table III). Preliminary experiments had suggested such an intercept, but this can now be excluded within the limitations of the data.

It is to be noted that the myothermal data providing the abscissa values were obtained on separate muscles. To allow the correlation, both these and the biochemical data were grouped in clusters as indicated in Fig. 1.
TABLE II
SUMMARY OF FINAL SERIES OF EXPERIMENTS (see text) ON THE
PHOSPHORYLASE $a$ AND PHOSPHORYLASE KINASE CONTENTS
AFTER 40 ANAEROBIC TWITCHES IN THE EXPERIMENTAL
MODES DESCRIBED IN THE TEXT

| Modes of activity | $n$ | Total phosphorylase | Phosphorylase fraction $a$ | Kinase activity ratio | Heat per 40 twitches | Heat (n) |
|-------------------|-----|---------------------|---------------------------|----------------------|---------------------|---------|
| Resting           | 11  | 49.8±8.1            | 0                         | 0.092±0.021          | 0                   | 0       |
| Stretched $L_o + 1.2$ cm | 12  | 53.4±13.2           | 0.095±0.015               | 0.079±0.026          | 29.4±6.0           | 4       |
| Lightly loaded    | 12  | 40.9±13.9           | 0.217±0.034               | 0.087±0.029          | 89.8±11.2          | 3       |
| Isometric (40)    | 14  | 47.3±12.7           | 0.344±0.027               | 0.097±0.013          | 91.5±13.5          | 4       |
| Isometric (8+32)  | 11  | 55.6±12.2           | 0.301±0.024               | 0.096±0.020          | 153.8±26.5         | 5       |
| Maximum loaded $0.4$ $P_o$ | 14  | 47.1±12.5           | 0.384±0.043               | 0.089±0.024          | 133.8±26.5         | 5       |
| Average           |     | 49.0±12.1           |                           | 55.0                 |                     |         |

Figure 1. The linear regression between the energy turnover per cycle (abscissa) and the percent phosphorylase $a$ activation (ordinate). Described in the text under Variation of Working Conditions. The separately grouped biochemical and myothermal data are shown on Table II, for each point on the graph. The diamond-shaped data point at 91.5, 0.301 represents the isometric experiments (8+32) of Table II. The solid line is the regression derived from these data, the broken line is derived from the data of Fig. 2.

Variation of Isometric Force
In the second experimental protocol, all contractions were isometric, and the energy output was varied by stretching the muscles so as to diminish filament overlap, as in the myothermal investigations of Smith (15) and Homsher et
Stretches from one experiment to the next were varied by 3-mm steps, but due to the variability of the muscles and the relative arbitrariness of the definition of $L_s$, no clustering of points resulted. However, since in this design each muscle allowed determination of its own independent variable, i.e. the relative force $P/P_o$, all results could be entered individually. Fig. 2 represents the data as obtained, whereas Table III contains the regression equation for the dependence of phosphorylase activation upon $P/P_o$.

Through comparison with the measurements of Homsher et al. (5, their Figure 7) it is possible to transform the primary data of Fig. 2 into a relation between phosphorylase activation and the energy per contraction cycle. This regression (Table III, and entered into Fig. 1 as the broken line) is closely comparable to that derived from the other experimental designs, which was obtained on the same animal population.

An interesting cross check is provided by the following comparison. The regression of phosphorylase upon relative tension has the firmly established intercept of $0.0864 \pm 0.0118$ phosphorylase $a$ (Table III). This is because at $P/P_o = 0$, the mechanical response but not the excitation-contraction coupling mechanism has been eliminated. This intercept is in excellent agreement with the mean value of $0.0954 \pm 0.0153$ for mode ii (Table II).
**DISCUSSION**

The results described deal with features of the regulation of metabolism in muscle. In a steady state, ATP is generated aerobically and from blood-borne fuel. In activity beyond the steady-state level, anaerobically or in isolation, a muscle is a closed system and uses its stored glycogen. Our work invites discussion of two aspects: the rate-limiting step at rest, and the mechanism of activation upon contractile activity.

The rates of intrinsic metabolism at rest and in maximal activity may vary as much as a thousandfold, and at the upper level no steady state may be possible. Even a resting frog muscle, anaerobically, loses phosphoryl creatine, suggesting that the resting glycolysis rate is limited not by ATP usage but by a step in the glycolytic pathway (16, pp. 38–39), and many considerations point to phosphorylase (17). This situation was not satisfactorily understood as long as the phosphorylase a fraction was thought to be 0.20 (11) and even the improved value of 0.01 to 0.05 (8) still leaves an excess of active enzyme as we shall see.

The numerous phosphorylase assays performed in this study give explicit information as to the "maximal capacity" (17) of phosphorylase. This is of the order of 8–10 μmol/g/min at 20°C. The resting glycolysis in frog muscle at 20°C is 0.035 μmol/g/min. Thus, the discrepancy is about 250-fold, i.e., a phosphorylase-a fraction of 0.004 would suffice to account for the resting glycogenolysis. The limits of our analytical techniques in the assays do not allow us to detect nor to exclude this fraction with certainty. This encourages the inquiry of how much phosphorylase b activated by adenylate would con-
tribute, in the presence of presumable amounts of inhibiting ATP and glucose-6-phosphate, and of activating AMP (19, 17). From investigations by Parmeggiani and Morgan (19), and accepting our AMP concentration (20) of 0.03 mM, one would estimate that the catalytic rate of phosphorylase \( b \) would be of the order of 0.001 of the full rate, and less than that if part of the AMP were bound to other enzymes (21). Thus, while the nature of the information allows no further precision, it seems that there is no gross discrepancy in explaining the rate-limiting position of the enzyme in resting anaerobic frog muscle. Our data allow that either an undetectable \( a \) fraction or an activated phosphorylase \( b \), or a combination of both, would suffice to account for the resting glycolytic rate, and that neither one is indicated to be in obvious excess. The large discrepancy found by Fischer et al. (17) is in part due to the assumption of a much higher AMP concentration.

The second point of our work is the finding that phosphorylase activation is dependent upon the energy turnover per cycle. Our conclusions are the opposite of those by Danforth and Helmreich (22) and we must therefore examine their data to see if they are not in reality compatible with our findings. They are, taking recourse to the results of investigations on energetics (Mommaerts, 23; Homsher and Rall, 6). The main issue is that at the temperature of their experiments, there is no Fenn effect (Homsher et al., 24; Mommaerts, 25). In detail, there are the following points: (a) In their Fig. 1 stimulation at 20°C at six per second gives more activation and sooner than at two per second; the averaged rate of energy liberation is three times greater in the former case. (b) In their Fig. 3, the energy liberation was varied by changing the afterload at four contractions per second; for this case, the total energy liberation is quite constant at 20°C, thus their finding is as expected (see Homsher et al. [5], Fig. 7). (c) In their Fig. 5, phosphorylase activity is a function of stimulus frequency and thus of energy per unit of time. (d) In their Fig. 7, phosphorylase \( a \) depends on the external K⁺ concentration as does the energy turnover as measured, e.g., by the Solandt effect. Thus we conclude that there is no factual discrepancy with the results of Danforth and Helmreich, and that their opposite conclusion stems from the insufficient understanding at the time of the energetics of contractions under different loading conditions. In our investigation, the work conditions were precisely specified, and were expressed quantitatively on the basis of heat measurements performed for the purpose.

Two kinds of experiments were performed by the designs detailed in the experimental section, both effecting a considerable range of variation of the energy per twitch. The second design has the merit of a free choice of the independent variable, the former has the merit of showing that the results seem independent of the mode in which the variation is brought about. The results of both were the same: the formation of phosphorylase \( a \) is proportional
to the energy turnover per twitch. Thus, the muscle cell has means to assess the energy turnover that occurs, and to use this as the signal determining phosphorylase activation just like it uses it to determine restitution respiration.

The connection between phosphorylase \( a \) and energy per 40 twitches (and, by inference, per twitch) is of the form \( a = mx \), in which \( x \) is the energy in millicalories. The two different designs of experiments gave the same slopes, \( m = 0.00317 \) and \( 0.00311 \). (See Table III.) As was shown in the experimental part, we conclude that there is no intercept. This is of physiological importance as showing that the energy turnover per twitch is the sole determinant, no additional significance attaches to any one feature, as would be the case if e.g., the occurrence of excitation or of the calcium cycle would make a special and separate contribution.

The third point of our findings requires some explicit discussion in the light of present views on the nature of phosphorylase kinase (12, 18, 26). This enzyme occurs in two forms which can be called inactive and active with respect to their behavior at pH 6.8 at which the former is ineffective, while at pH 8.2 both function comparably. The transition or activation is due to phosphorylation by a protein kinase which is dependent upon cyclic adenosylate. Our findings show then that, unlike under adrenergic influence (10), phosphorylase \( b \rightarrow a \) transformation takes place without activation of phosphorylase kinase in that sense. Both forms of the kinase, at whichever pH, require calcium ions in small amounts for their action (27, 28, 29). Thus the activation occurring in conjunction with the excitation-contraction cycle is likely to be pulsatile, in that the cycling calcium distributes itself between the troponin system on the one hand, phosphorylase kinase on the other hand, the latter temporarily reaching whatever level of activity results at the given pH and state of phosphorylation.

This leaves, however, the major question of the regulatory connection between the energy turnover per cycle and the phosphorylase transformation eventually resulting. One could propose a relation between the energetics and the amount of calcium cycling under different work conditions. However, the current development of the field (5) is taking place without reference to such a relation, and no indications have appeared to the contrary. Unless we learn otherwise, we must therefore consider that the pulsatile kinase activation is, in turn, modulated by other signals.

The nature of this signal may or may not be in common with other metabolic regulations, e.g. that of mitochondrial respiration by ADP. This mechanism is not as impossible as it seemed when the best estimates of ADP (not bound to actin) were 0.25 mM (Seraydarian et al., 30). Of this amount, it may now be assumed that about 0.20 mM is bound to myosin in the state of rest (31, 32). What remains would be compatible with the creatine kinase equilibrium as analyzed by Carlson and Siger (33) to be of the order of 0.03
mM, so that changes in concentration too small to be analytically detectable would be significant in proportion to actual levels. Also, every initiation of a contraction cycle would passingly unload 0.2 mM ADP which would persist briefly until rephosphorylated by creatine kinase. So seen, a signaling control function of ADP with respect to metabolism is now plausible, but there is no connection at this time to the mechanism of phosphorylase kinase.

A solution of this problem would be of importance because the regulatory influence of the phosphorylase activating system may not be limited to metabolic control only. We mention the phosphorylation of troponin, although this has not been shown to be of functional interest,\(^1\) of sarcomentric protein likely to be crucial in inotropic responses (Katz [34]; La Raia and Morkin [35]), and finally we point to the problem of hypertrophy which is also in response to a measure of the integrated energy turnover level.

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