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Calcium-Dependent Regulation of Phosphorylase Activation in a Fast-Twitch Oxidative-Glycolytic Skeletal Muscle

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SUMMARY

Calcium-mediated phosphorylase kinase activation has been studied in the rat flexor digitorum brevis, a fast-twitch oxidative-glycolytic skeletal muscle that exhibits a robust inward Ca^{2+} current [*Can J. Physiol. Pharmacol.* 63:958–965, 1985]. This system provided an opportunity to compare the regulation of contraction and activation of phosphorylase by extracellular and intracellular sources of Ca^{2+}. In muscles repetitively stimulated at 21°, there appeared to be a close correlation between the control of contraction and phosphorylase activation. Blocking extracellular Ca^{2+} entry promoted an inactivation of phosphorylase and diminished the elevation of resting tension, which in untreated muscles ensues with the onset of fatigue. The response of muscles stimulated at 37° was in distinct contrast. Phosphorylase, following initial rapid activation, was then briskly inactivated despite the continuation of a near-maximal contractile response. An elevation in resting tension during stimulation was observed at 37° but was a transitory response in comparison to what was seen at 21°. Blocking the entry of external Ca^{2+} inhibited this response. Sarcolemmal Ca^{2+} channel blockers had no effect on the observed phosphorylase response at 37°, but phosphorylase was already nearly fully inactivated before their effects were manifested on contraction. Thus, at this temperature there is a clear dissociation between Ca^{2+}-mediated regulation of contraction and the production of metabolic energy by enhanced glycogenolysis. This appears to occur because, although Ca^{2+} induces phosphorylase activation, a subsequent, but rapid non-Ca^{2+}-mediated event promotes inactivation, even while Ca^{2+}-mediated contraction is being sustained.

The activation of phosphorylase in skeletal muscle during contraction is well documented and results from the allosteric activation of phosphorylase kinase by Ca^{2+} (1–4). Since activation of this enzyme *in vivo* occurs at a Ca^{2+} concentration(s) which also initiate(s) contraction (5), regulation by Ca^{2+} has the net effect of providing both for contraction and for the energy necessary to sustain contraction. Although it is clear that the SR is the primary source for this Ca^{2+}, some studies have noted a potential role for extracellular Ca^{2+} (6–8). Ca^{2+} enters the cell primarily through voltage-dependent channels in the sarcolemma in what is termed an "inward" Ca^{2+} current. Although the significance of this source of Ca^{2+} is controversial (9), it is proposed to affect a number of processes. The regulation of cellular activity via the influx of extracellular Ca^{2+} is well established in cardiac muscle and appears to contribute to the level of Ca^{2+} within the cytosol as well as to provide a stimulus for further Ca^{2+} release from the SR (10–12). In skeletal muscle, extracellular Ca^{2+} has been suggested as a possible link between sarcolemmal depolarization and SR Ca^{2+} release, but it may also contribute to the regulation of muscle function during periods of sustained contraction (6, 13–15).

We have recently described an *in vitro* mammalian muscle system (15) which offers the possibility of exploring the functional role of inward Ca^{2+} currents. The FDB is a homogeneous muscle (>90% FOG fibers) located on the plantar surface of the rat hindfoot. Of particular interest, the FDB responds during intermittent repetitive stimulation by developing an increase in its baseline or resting tension. The increase in baseline appears to be a result of inward Ca^{2+} currents moving through voltage-dependent Ca^{2+} channels since these currents may be modified by preincubation in media without Ca^{2+} or with agents that block voltage-dependent channels. The present study describes the use of the FDB muscle preparation to examine the contribution of extra- and intracellular Ca^{2+} to the regulation of Ca^{2+}-dependent phosphorylase kinase activation and the subsequent activation of phosphorylase.

Materials and Methods

Animals. Female Sprague-Dawley rats weighing 230 ± 5.5 g were kept on a 12-hr light cycle and given Purina Rat Chow *ad libitum*. Animals were kept in this environment for at least one full day prior

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ABBREVIATIONS: SR, sarcoplasmic reticulum; FDB, flexor digitorum brevis; FOG, fast-twitch oxidative-glycolytic fibers; FG, fast-twitch glycolytic fibers; SO, slow-twitch oxidative fibers; MES, 4-morpholineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

to experimentation. Rats were anesthetized by an intraperitoneal injection of chloral hydrate (35 mg/100 g body weight) and the FDB muscles were removed. A 4-O suture was tied around the proximal tendon and the tendon was sectioned just proximal to the suture. Following dissection to free the muscle from underlying tissue, the distal tendons were cut and the muscle was placed directly into the incubation chamber. The distal tendons were held in a fixed clamp and the proximal tendon was tied directly to a Grass FT-03 force transducer using 4-O suture.

Incubation studies. The incubation medium consisted of 150 ml of normal Liley's solution (NaCl, 137 mM; KCl, 5 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM; NaH_2PO_4, 1 mM; and glucose, 11 mM, pH 7.3–7.4), maintained initially at 21 ± 1°, and continuously gassed with 95% O_2/5% CO_2. In some experiments, a modified Liley's solution containing verapamil hydrochloride (Knoll Pharmaceutical) or 3 mM $CoCl_2$ was added to the incubation chamber. Verapamil was first dissolved in 0.01 N HCl and then added to normal Liley's solution to give the desired final concentration. When $CoCl_2$ was added, the Liley's solution was prepared by omitting the NaH_2PO_4 to avoid precipitation of $Co_3(PO_4)_2$.

Stimulation procedure and contractile measurements. The stimulation procedure was essentially that described by Carlsen *et al.* (15), using a paradigm modified from Burke *et al.* (16). The muscle was stimulated directly via a Grass EMG electrode inserted into the proximal tendon. The negative lead was attached to the clamp holding the distal tendons. After adjustment of the muscle to the length at which twitch tension was maximal, the following contractile measurements were obtained: (a) maximum isometric twitch tension; (b) time to peak twitch contraction (T_c); (c) half-relaxation time ($T_{\frac{1}{2}R}$); (d) peak isometric tetanic tension (at 30 or 50 Hz). The muscles were equilibrated at the desired temperature for 20 min and continuously stimulated at 0.05 Hz (0.5-msec pulse width), except for the $CoCl_2$ group where no equilibratory stimulation was used. After equilibration, the contractile measurements were repeated and the muscles stimulated with interrupted tetanic trains (at either 30 or 50 Hz, 330 msec/train, 1 train/sec) for the indicated period. Muscles which were not stimulated following equilibration were used as controls for each group. At the end of the desired stimulation period, the incubation medium was rapidly drained and the muscles were freeze-clamped using aluminum tongs cooled in liquid N_2. Muscles were frozen within ~1 sec of the last contraction and stored at −70° for later analysis.

Tissue extraction and enzyme assay. Individual muscles were powdered, following precooling in liquid N_2, in a stainless steel percussion mortar cooled in dry ice. When tissues were to be analyzed for both phosphorylase kinase and phosphorylase, three to four similarly treated muscles were pooled to provide enough powder for the multiple assays. Phosphorylase was assayed by a modification of the procedure of Hardman *et al.* (17). Briefly, about 50 mg of frozen muscle powder were rapidly homogenized in 0.5 ml of extraction buffer at 0° using a glass-Teflon Potter-Elvehjem homogenizer. The extraction buffer contained 50 mM MES (pH 6.1), 100 mM NaF, 5 mM EDTA, 45 mM 2-mercaptoethanol, 1 mM PMSF, 100 μM TPCK, and 2 mM benzamidine. The homogenate was centrifuged at 12,000 × *g* for 10 min at 4° and the supernatant was filtered through glass wool. The assay reaction contained 20 μl of muscle extract in 2.0 ml of 25 mM potassium phosphate at pH 7.0, 15 mM 2-mercaptoethanol, 4 mM EDTA, 6 mM magnesium acetate, 50 mM NaF, 0.167% (w/v) glycogen, 0.17 mM NADP, 6.4 units of glucose-6-phosphate dehydrogenase, and 28.8 units of phosphoglucomutase in the presence or absence of 2 mM 5′-AMP. The formation of NADPH at 30° was followed using a dual beam spectrophotometer (Aminco) and was linear for about 20 min. Phosphorylase activity was expressed as a ratio of its activity ± AMP. Phosphorylase kinase was assayed as described previously (18) based on the incorporation of ^{32}P into phosphorylase at 30°, with the results reported as a ratio of activities at pH 6.8/pH 8.2.

Data analysis. Statistical significance between group means was determined using analysis of variance with specific differences at individual time points evaluated using Duncan's New Multiple Range Test.

Results

Contractile response. Intermittent repetitive stimulation of FDB *in vitro* leads to a progressive rise in baseline (resting) tension. The increase, as noted previously (15), is modulated by muscle temperature and stimulus frequency, and may be depressed by preincubation with agents that block voltage-dependent inward Ca^{2+} currents. In the present study, the most profound increase in baseline tension was generated in muscles maintained at 21° and stimulated with 30-Hz trains lasting 330 msec and repeated 1 per sec for 2 min (Fig. 1, Table 1). The increase in baseline tension accounted for 61% of the tension generated during the final stimulus train. Thus, whereas total tension declined by only 19% during the 2-min stimulation, the majority of maintained tension was due to a rise in baseline tension.

Preincubation in either verapamil (10^{-4} M) (Fig. 1, Table 1) or Co^{2+} (3×10^{-3} M) (Table 1) reduced the stimulation-induced increase in baseline tension by more than 60%. Both agents reduced initial train tension, but Co^{2+} had the greatest effect. Co^{2+} also reduced final train tension, whereas verapamil had no significant effect on the tension of the final train. The ratio of final train tension to initial train tension is characterized as the fatigue index (16). The fatigue index was 0.31 ± 0.03 for control FDB and 0.32 ± 0.02 for verapamil-treated muscles. In contrast, the fatigue index for Co^{2+}-treated muscles was 0.23 ± 0.07. A comparison of these fatigue indices suggests that verapamil affects only inward Ca^{2+} currents whereas Co^{2+} reduces both the inward Ca^{2+} current and the release of Ca^{2+} from the SR during excitation-contraction coupling.

Raising the temperature of the incubation medium to 37°

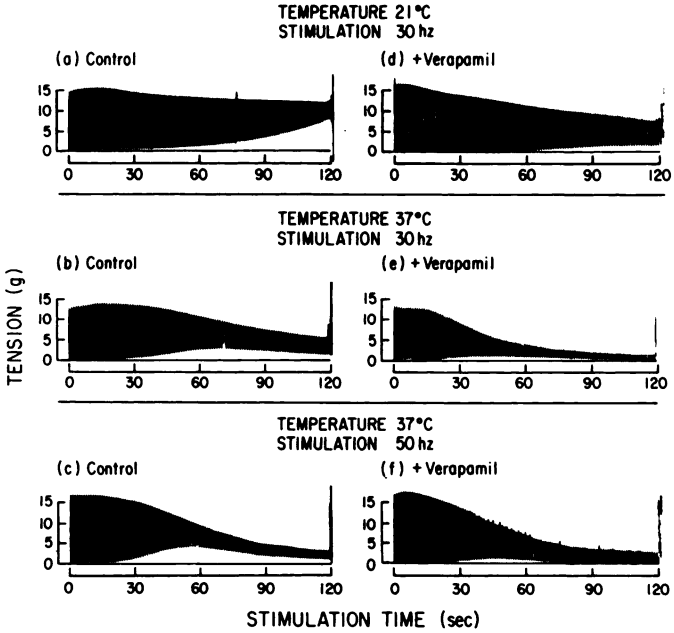

Fig. 1. Time course of tension development during 2 min of electrical stimulation in untreated and verapamil-treated isolated FDB muscles at 21°and 37°. Muscles were stimulated as described under Materials and Methods and the legend to Table 1 at the indicated temperature and frequency of stimulation in either the absence (a–c) or presence of 10^{-4} M (d) or 10^{-5} M (e and f) verapamil. Recorder tracings are representative of the pattern of tension development under each condition.

TABLE 1

Effects of verapamil, Co^{2+}, and incubation temperature on the contractile properties of isolated rats FDB muscle before and after electrical stimulation

Muscles were removed from the rat and mounted in the incubation chamber as described under Materials and Methods. Initial contractile measurements were made following a 20-min equilibration period during which time the muscles were stimulated at 0.05 Hz to produce single twitches (except for the $CoCl_2$ group where no equilibratory stimulation was given). Final contractile measurements were made after the muscles were stimulated by intermittent tetanic trains of stimuli at either 30 or 50 Hz for 330 msec, 1 train/sec for 2 min. Tension is reported in grams. Baseline tension is determined from the recorder output tracing as the distance between the original tension baseline and the lower limit of the oscillatory tension tracing (see Fig. 1). Total tension is measured from the original tension baseline to the upper point on the oscillatory tension tracing. Final train tension is the difference between the upper and lower points of the tension oscillation. Values are expressed as means ± standard error.

	Incubation temperature	Stimulus frequency	Initial train tension	N	Tension after 2 min of repetitive stimulation			N
					Total tension at 2 min	Increase in baseline tension	Final train tension	
	deg C	*Hz*						
Control	21	30	16.0 ± 0.2	83	12.9 ± 0.5	7.9 ± 0.9	5.0 ± 0.5	31
10^{-4} M Verapamil	21	30	14.6 ± 0.3*	56	7.3 ± 0.5*	2.4 ± 0.6*	4.9 ± 0.3	14
3×10^{-3} M Co^{2+}	21	30	9.9 ± 0.5*	15	4.8 ± 0.6*	2.9 ± 1.1*	1.9 ± 0.5*	4
Control	26	30	14.8 ± 0.7	29	9.8 ± 0.9	3.7 ± 1.6	6.1 ± 1.1	8
Control	37	30	12.1 ± 0.8	17	3.9	1.2	2.7	2
10^{-5} M Verapamil	37	30	11.8 ± 0.7	18	1.7	0.3	1.4	2
Control	37	50	15.3 ± 0.6	23	3.4 ± 0.4	1.4 ± 0.2	2.0 ± 0.4	7
10^{-5} N Verapamil	37	50	14.9 ± 0.7	16	3.5 ± 0.6	0.3 ± 0.2	3.2 ± 0.5	5

* $p < 0.05$ difference from corresponding control group.

depressed the stimulation-induced increase in baseline tension (Fig. 1, Table 1). Baseline tension rose by 1.2 g in control FDB stimulated at 30 Hz (unfused tetanus) and by 1.4 g in muscles stimulated at 50 Hz (fused tetanus). The increases were less than 20% of the maximum increase obtained at 21°. The pattern of baseline increase was also different at the two incubation temperatures. At 21°, baseline tension started to increase approximately 45–60 sec into the stimulation period and rose progressively as stimulation continued. At 37°, baseline tension began to increase earlier, starting approximately 30 sec after the initiation of stimulation. The tension increase, however, reached a peak during stimulation and then slowly declined during the remainder of the 2-min period.

Verapamil (10^{-5} M) produced a proportionately similar reduction in the stimulation-induced increase in baseline tension at 37° as compared to 21°. Verapamil depressed the rise in baseline tension by 70%, relative to control, at 21°, by 75% at 37° with 30 Hz stimulation, and by 79% with 50 Hz stimulation (Table 1). The increase in baseline tension contributed 41% of total tension in the final train in FDB at 37° stimulated at 50 Hz, but only 9% of total tension in muscles preincubated in verapamil.

Phosphorylase activation during FDB contraction. Ca^{2+}-dependent activation of phosphorylase, occurring via an allosteric activation of phosphorylase kinase, has been well established by both *in vivo* and *in vitro* investigations (19). The observations obtained using FDB provide an opportunity to explore the possible contributions of extracellular and intracellular Ca^{2+} sources to phosphorylase activation during muscle contraction. The measurement of phosphorylase activity also offers an alternate means of assessing changes in cytosolic free Ca^{2+}. Repetitive stimulation of control FDB at 21° caused a rapid increase in phosphorylase *a* activity that peaked at about 60 sec and remained elevated near its maximal level for the remainder of the 2-min stimulation period (Fig. 2a). This pattern of response was markedly altered in the presence of 10^{-4} M verapamil (Fig. 2a). Stimulation after preincubation in verapamil still generated essentially the same maximal level of phosphorylase *a* activity at 60 sec, but the rate of increase in activity was significantly slower. Moreover, upon reaching peak activation, phosphorylase was rapidly inactivated during the remainder of the stimulus period. At 2 min, activity in the presence of verapamil had decreased to approximately 40% of peak activity, whereas it was 76% of peak in controls.

Preincubation in Co^{2+} reduced both the rate of rise of phosphorylase *a* activity and the level of maximal activity (Fig. 2a). Peak activity was reduced by 40% compared to control FDB and by 34% compared to verapamil-treated muscles. Activity in Co^{2+}-treated muscles declined during the second minute of stimulation and by 2 min was at a level similar to that reached by verapamil-treated muscles. The percentage reduction in phosphorylase peak activation with verapamil and Co^{2+} showed a quantitative correlation with the percentage reduction that was induced in the initial train tension; i.e., verapamil reduced peak activation by 8% and initial tension by 9%, whereas Co^{2+} reduced peak activation by 40% and tension by 38%. The correspondence between activities at the end of the 2-min stimulation in Co^{2+} and verapamil showed a proportional relationship to their effect on the rise in baseline tension. Both agents reduced the rise in baseline tension by approximately 66% and phosphorylase activity by approximately 54%.

To confirm that the observed activation of phosphorylase is via Ca^{2+}-dependent allosteric activation (as expected) rather than covalent modification of the kinase (as produced by cAMP), phosphorylase kinase activity was also determined. The *inset* in Fig. 2a indicates that the phosphorylase kinase pH 6.8/8.2 activity ratio remained at basal levels throughout the 2-min stimulation. It was also unaffected by incubation with verapamil (10^{-4} M).

Muscles incubated at 37° and stimulated at either 30 or 50 Hz generate phosphorylase activation profiles that are quite distinct from those obtained at the lower temperature (Fig. 2, b and c). Intermittent stimulation at either frequency produced a rapid initial activation followed by inactivation that began within 10–15 sec of the initiation of repetitive stimulation. Verapamil did not appear to significantly modify this profile of activation (despite its subsequent substantial effect on baseline tension), suggesting that either there was no contribution of

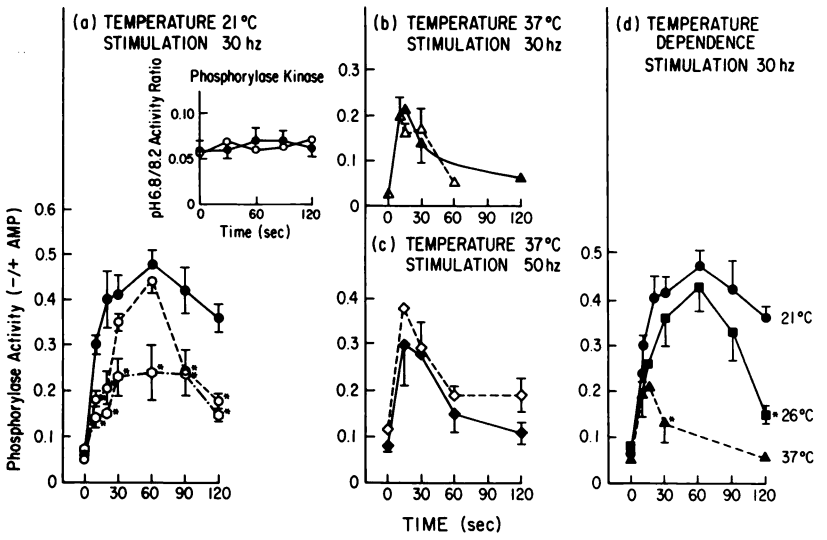

Fig. 2. Time course and temperature response of phosphorylase activity in isolated untreated and drug-treated FDB muscle. Muscles were stimulated for various lengths of time as described under Materials and Methods and the legend to Table 1. Total phosphorylase activity averaged 0.17 μmol·mg protein^{-1}·min^{-1} and was not affected by electrical stimulation or drug treatment. a. Incubation at 21° at 30 Hz: ●, untreated; ○, verapamil, 10^{-4} M; ○, $CoCl_2$, 3×10^{-3} M. b. Incubation at 37° at 30 Hz: ▲, untreated; △, verapamil, 10^{-5} M. c. Incubation at 37° at 50 Hz: ◆, untreated; ◇, verapamil, 10^{-5} M. d. Temperature dependence of the time course of phosphorylase inactivation in untreated FDB muscles; data for 21° and 37° are replotted from a and b. Values expressed as mean ± standard error represent $N = 3$–9 observations. Points without standard error bars are the means of duplicate measurements. *, $p < 0.05$ compared to corresponding untreated groups. The *inset* in a shows the activity of phosphorylase kinase during the course of stimulation, measured as described under Materials and Methods, symbols as in a.

the inward Ca^{2+} current or, possibly more likely, that whatever was causing the strong degree of inactivation dominated the response seen and masked any possible contribution. Since the effect on baseline tension is not seen until at least 30 sec into the stimulation time course, the apparent lack of effect of verapamil on phosphorylase activation does not indicate that it and contraction were differentially regulated by external Ca^{2+} sources. What is apparent with stimulation at 37° (and not seen during the 2-min course of stimulation at 21°) is the dissociation of the Ca^{2+}-dependent regulation of phosphorylase and the control of contraction. This is illustrated by the data in Figs. 1 and 2, from which it can be seen that the rapid inactivation of phosphorylase at 37° occurs at a time when tension development is still maximally stimulated.

To permit further comparison, the control data of Fig. 2, a and b, have been re-illustrated in Fig. 2d, together with additional data for phosphorylase activation with muscles incubated at 26°. All of these data are for muscles stimulated at 30 Hz. The data show that as the incubation temperature was increased, the degree of inactivation was enhanced and the time course of inactivation was more rapid, Thus, at 21° minimal to no inactivation had occurred by 2 min of stimulation, whereas at 37°, phosphorylase activity had returned to near basal levels between the first and second minutes of stimulation. (At 21°, phosphorylase is inactivated with stimulation periods longer than 2 min and it returns to near basal levels with 4 min of repetitive stimulation; data not shown.) When these data are compared with the contractile properties, there appears to be a correlation between the overall extent of phosphorylase activation and the duration of the Ca^{2+} pulse. If the combined values of $T_c + T_{\frac{1}{2}R}$) are taken as an indication of the duration over which cytosolic Ca^{2+} levels are elevated,[1] then as twitch time diminishes and, thus, there are longer periods of basal Ca^{2+} levels, the extent of phosphorylase activation appears likewise to be diminished. This suggests that elevated Ca^{2+} could be the trigger for phosphorylase activation but that inactivation is likely to be Ca^{2+} independent.

Temperature dependence of verapamil action. The concentration of verapamil used in these studies (Figs. 1 and 2) is severalfold higher than that required to block Ca^{2+} channels in cardiac muscle (20), but within the concentration range that others have found necessary to block skeletal muscle sarcolemma Ca^{2+} transport (8, 21). In the studies reported here, it was found that, whereas 10^{-5} M verapamil was effective with muscles incubated at 37°, a 10-fold higher concentration was necessary with muscles incubated at 21°. These are the concentrations used in the experiments presented in Figs. 1 and 2 and in Table 1, and this concentration dependency is illustrated in Fig. 3. At 10^{-5} M verapamil, with muscles incubated solely at 21°, there was no effect on phosphorylase activation and only a small effect on contractile baseline tension, whereas at 10^{-4} M verapamil, both were substantially reduced. The reason for this difference in dose response at the two temperatures is unknown but may relate to an increase in "solubility" of verapamil into the muscle cell membrane at the higher temperature (and fluidity). It has been suggested that, to be effective in blocking Ca^{2+} channels, verapamil must reach the cytosolic surface of the Ca^{2+} channel protein (22). Since at higher concentrations verapamil has other actions, one of which is a local anesthetic effect resulting in diminished SR Ca^{2+} release and a severely reduced contractile response, the basis for this tem-

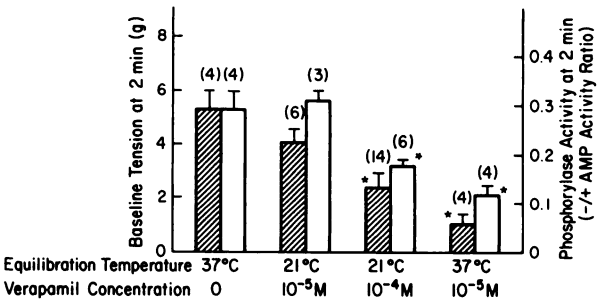

Fig. 3. Effect of incubation temperature on verapamil action. Muscles were first equilibrated at the indicated temperature and verapamil concentration for 20 min. The bath temperature was then reduced to 21° and the muscles were incubated for an additional 15 min. Muscles were then stimulated with interrupted repetitive trains at 30 Hz for 2 min as described under Materials and Methods. Values are expressed as means ± standard errors. *, $p < 0.05$ compared to untreated group. ■, measurements of contractile tension; □, measurements of phosphorylase.

[1] The combined value of $T_c + T_{\frac{1}{2}R}$ will be closely proportional to the length of time that Ca^{2+} is elevated but not quantitatively identical. The T_c = 72.5 ± 0.9 msec and $T_{\frac{1}{2}R}$ = 44.6 ± 1.2 msec (N = 40) at 21°; T_c = 46.8 ± 2.3 msec and $T_{\frac{1}{2}R}$ = 36.4 ± 2.1 msec (N = 11) at 26°; T_c = 26.9 ± 1.0 msec and $T_{\frac{1}{2}R}$ = 42.8 ± 3.3 msec (N = 16) at 37°.

perature dependency was further investigated. Experiments were performed in which muscles were first incubated at 10^{-5} M verapamil at 37°, following which the muscle bath was equilibrated to 21°, and the muscles were electrically stimulated at this temperature. Under these conditions, 10^{-5} M verapamil was now at least as effective in diminishing both baseline tension and phosphorylase activity as was 10^{-4} M verapamil in muscles incubated and stimulated at 21°. A concentration of 10^{-5} M verapamil is similar to that which others have shown to be effective in blocking skeletal muscle sarcolemma Ca^{2+} channels (21).

Discussion

This study represents an effort to distinguish the contributions of two sources of Ca^{2+} to the activation and inactivation of phosphorylase during moderate muscle activity. There seems to be little doubt that the Ca^{2+} required for the initial allosteric activation of phosphorylase kinase and the consequential activation of phosphorylase is provided predominantly by the SR. The activation of phosphorylase at 37° was the same in both control and verapamil-treated muscles. Moreover, peak activity levels were correlated with tension, being greater in muscles stimulated at 50 Hz than in those stimulated at 30 Hz. Similarly, the reduction in rate of activation and peak activity in verapamil- and Co^{2+}-treated muscles stimulated at 21° was associated with a proportionate reduction in tension, but, nevertheless, maximum activation was not markedly affected by verapamil addition. However, inward Ca^{2+} currents present in this muscle did appear to affect the ability of the muscle to maintain phosphorylase activation. This was apparent in muscles stimulated at 21°. Reduction of inward Ca^{2+} currents with verapamil (or Co^{2+}) led to a more rapid and substantial inactivation of phosphorylase. The inhibition of inward Ca^{2+} currents presumably reduced accumulation of free Ca^{2+} in the sarcoplasm, as suggested by the reduced increase in baseline tension and twitch half-relaxation time (data not shown) at the end of the stimulation period. The explanation for phosphorylase activation and inactivation based on the data from FDB muscles stimulated at 21° thus appears straightforward. Phosphorylase was activated by Ca^{2+}, released principally from the SR, and the activation was maintained by a significant contribution from inward Ca^{2+} currents. However, this description is not complete since phosphorylase inactivation was seen in muscles stimulated at 37°, whereas tension was maximal, regardless of the presence or absence of inward Ca^{2+} currents. Likewise, inactivation occurred at 21° if stimulation was prolonged for 4 min despite only a minor decrease in total contractile force (data not shown).

The dissociation between phosphorylase activation and contractile activity has been previously described in other skeletal muscles (23, 24). Conlee *et al.* (23) studied phosphorylase activation in rat plantaris muscle, which contains a near-equal mixture of FG and FOG fibers, and in rat soleus, which is composed primarily of SO fibers. The time courses of phosphorylase activation and inactivation in plantaris muscle were very similar to those seen here with FDB (23). With soleus, phosphorylase inactivation was only seen after some minutes of stimulation. What accounts for the dissociation of these two Ca^{2+}-mediated processes (i.e., phosphorylase activation and contraction) is not known. Possible explanations include that the two are mediated by different Ca^{2+} pools (this is probably unlikely given the results obtained here with verapamil), or that a given concentration of free Ca^{2+} must be present for a particular duration in order to maintain phosphorylase activation. A possibility to be considered is that a Ca^{2+}-mediated inactivation of phosphorylase may occur as an independent secondary response, catalyzed by a protein phosphatase such as calcineurin. This enzyme is present in skeletal muscle (25), although it is not associated with the glycogen particle where, presumably, most phosphorylase inactivation occurs. However, phosphorylase associated with the particle might be available for dephosphorylation by a cytosolic phosphatase (26) or, as glycogenolysis ensues, phosphorylase may dissociate from the glycogen particle (27) and then be dephosphorylated. The rapid inactivation of phosphorylase at 37° could possibly be accounted for by such a mechanism; however, if this is so, it is curious that at 21° extensive inactivation is observed in the presence of verapamil when cytosolic Ca^{2+} is presumably diminished. Most likely some other explanation accounts for the divergence of control seen in the profiles of contraction and phosphorylase activity, and phosphorylase inactivation is a Ca^{2+}-independent phenomenon.

It is possible that the artificial nature of electrically stimulating a muscle *in vitro* could result in the inactivation of phosphorylase due to the absence of other physiological stimuli and that it does not occur under more physiological conditions of exercise. Richter *et al.* (24) have shown that in the soleus muscle of electrically stimulated perfused rat hindquarters, the transient nature of phosphorylase activation was abolished when epinephrine was added. Thus, with epinephrine present, the allosteric activation of phosphorylase kinase by Ca^{2+} is supplemented by a concomitant cAMP-induced covalent activation. Covalent modification of phosphorylase kinase, however, lowers the concentration of Ca^{2+} that is required for activation (5).

Other potential events which could result in an inability to maintain phosphorylase activity are a depletion of muscle glycogen and ATP levels. With regard to muscle glycogen levels, Constable *et al.* (28) have recently shown that phosphorylase activation in rat plantaris muscle is markedly inhibited 25 min after exhaustive treadmill exercise. In their study, treatments that increased or decreased muscle glycogen levels following exercise did not have any significant effect in either diminishing or enhancing the inhibition of phosphorylase activation. Thus, although the results of that study could not rule out that the depletion of muscle glycogen and the resulting disorganization of the physiological glycogen-protein complex played a role in the transient nature of phosphorylase activation, the authors suggested that others factors had to be involved. Similarly, in previous studies in which ATP levels were followed, phosphorylase inactivation was likewise observed despite well maintained cellular ATP concentrations (23, 24). In the present study, although ATP levels were not measured, they are not believed to be the cause of the inactivation of phosphorylase seen at 37° since muscle tension (another highly ATP-dependent process) did not begin to decline significantly until 15–20 sec after the marked decrease in phosphorylase activity.

In conclusion, the present study shows that at low, nonphysiological temperatures, the increase in baseline muscle tension and the allosteric activation of phosphorylase kinase, and the subsequent increase in phosphorylase activity, occur as a result of Ca^{2+} contributed not only from SR stores, but also from

extracellular sources via an "inward" Ca^{2+} current. However, particularly at physiological temperatures, there appears to be a dissociation of the regulation of phosphorylase activation and contractile activity due to factors which are, as yet, unclear but likely are independent of cytosolic Ca^{2+} and predominate over the Ca^{2+} stimulus of the activation reaction.

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