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Temporal Relationships between Isometric Force, Phosphorylase, and Protein Kinase Activities in Vascular Smooth Muscle (42008)

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Abstract. Vascular smooth muscle contractility is tightly coupled to ATP production by intermediary metabolism. To elucidate mechanisms underlying coordination of metabolism and contractility we studied the time course of isometric force, and the activation of phosphorylase and cAMP-dependent protein kinases during stimulation of bovine coronary arterial strips with KCl. Isometric force reached a maximum after 10 min of exposure to 30 m*M* KCl (ED_{90}) and was sustained throughout the subsequent 20-min period of contraction. In contrast, activation of phosphorylase was biphasic: enzymic activity reached a maximum (176 ± 10% of control) after 3 min of contraction and then, though remaining above control, activity declined to a lower level (135 ± 7% of control). However, no change occurred in the activity ratios for cAMP-dependent protein kinase assessed in either the presence (type II isozyme) or absence (type I isozyme) of 0.5 *M* NaCl. These data suggest that the activation of phosphorylase during K^+-induced contraction is independent of the cAMP system. The biphasic activation of phosphorylase may reflect transient changes in the intracellular concentration of Ca^{2+} or the activation of a phosphatase(s) during the response.

Phosphorylase, a key regulatory enzyme in glycogenolysis, is activated when vascular smooth muscle (VSM) is contracted by a variety of agonists *in vitro* (1–3). Namm (1) suggested that activation of phosphorylase during contraction of VSM, like its activation in skeletal muscle (4), results from stimulation of phosphorylase kinase by the increase in cytosolic Ca^{2+} associated with contraction. The activity of phosphorylase kinase in skeletal muscle can also be modified by cAMP-dependent protein kinase (5, 6). The mechanism whereby activation of the phosphorylase cascade occurs in smooth muscle, however, has not been completely elucidated. Such information is of interest because activation of phosphorylase may be an important functional link coordinating arterial metabolism and contractility (7–10). In this context, it is noteworthy that interaction between the contractile proteins, actin and myosin, can be modulated by Ca^{2+}-dependent phosphorylation of the regulatory 20,000-Da myosin light chains (10, 11) and that this phosphorylation can be modified by cAMP-dependent protein kinase (12–14). Thus, functional coordination between the glycogenolytic cascade and contraction–relaxation of smooth muscle may involve modulation of key enzymes by phosphorylation and dephosphorylation (7, 10, 15, 16).

In mammalian skeletal and cardiac muscle phosphorylase exists in an active phosphorylated form (phosphorylase *a*) and a less active dephosphorylated form (phosphorylase *b*). Activation of phosphorylase *b* in these muscles is mediated by phosphorylase kinase, an enzyme whose activity is dependent on Ca^{2+} and modulated by changes in its state of phosphorylation resulting from interaction with cAMP-dependent protein kinase (5, 6, 17). Recent studies on bovine coronary artery have shown that it contains both type I and type II isozymes of cAMP-dependent protein kinase (18). Moreover, β-adrenergic relaxation was associated with activation of the type II isozyme. The extent to which either of these isozymes participates in the activation of phosphorylase in VSM is unknown.

In this study we examined the time course of activation of phosphorylase and cAMP-dependent protein kinases during a K^+-induced isometric contraction in bovine coronary artery. The results showed that activation of phosphorylase followed a biphasic time course and that such activation was indepen-

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dent of cAMP-dependent protein kinase. The biphasic changes in activity may be related to similar biphasic changes noted for energy utilization (2, 19) and/or phosphorylation of the myosin light chains (20) suggesting that these events may be functionally coordinated.

Materials and Methods. Detailed descriptions including discussions of limitations and assumptions of the analytical procedures used in this study are available in recent publications from our laboratories (3, 8, 18). Therefore, only brief descriptions are included in this report.

Preparation of coronary arterial strips. Studies were performed with 87 bovine circumflex coronary arterial strips (12–15 mm long, 3–5 mm wide) prepared from 27 hearts. Two to four strips (12–15 mm long, 3–5 mm wide) were cut from a circular section of the artery and mounted in a muscle chamber for recording isometric tension (Grass, FT-03). The strips were stretched passively to optimal length by imposing a resting tension of 50 milliNewtons [mN, Refs. (18, 21)], and incubated in a physiological solution (20 ml) which was maintained at 37°C and aerated with 95% O_2 −5% CO_2 (pH 7.3–7.4). The composition of the salt solution (in m*M*) was NaCl, 130; $NaHCO_3$, 14.9; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4 \cdot H_2O$, 1.17; $CaCl_2$, 1.6; CaN_2 versenate, 0.026; and dextrose, 5.5. After 3 hr of equilibration the strips were challenged three to four times with an addition of KCl which incremented the bathing medium concentration by 30 m*M*. This concentration produced 90% of the maximal increase in isometric force attainable with KCl. As in previous studies, strips in which repeated responses to KCl did not agree within 10% of each other, or which failed to attain 90% of the minimum expected response (65 mN isometric force), were omitted from further study (18, 21). Acceptable strips were challenged with 30 m*M* KCl and frozen by rapidly lowering the muscle chamber and immersing (<2 sec) the strips in Freon 12 which had been precooled in liquid nitrogen. The frozen strips were utilized for assessment of phosphorylase and protein kinase activities (see below). Isometric force attained at the time of freezing was expressed as a percentage of the maximal force attained during the preceding previous test response to KCl. In all experiments, paired control strips (with 50 mN passive tension and no KCl challenge) were included from the same heart.

Phosphorylase activity measurements. Phosphorylase activity was determined in extracts of homogenates utilizing an enzyme-linked method as originally described by Hardman *et al.* (22) and modified by Paul (3). In the modified procedure, a continuous recording of the change in fluorescence at 455 nm (Perkins-Elmer 650-10S fluorescence spectrophotometer) provides a direct measure of the rate of NADPH and linked glucose-1-phosphate (G-1-P) formation, a measure of phosphorylase *a* activity. The rate of change in fluorescence following addition of 2 m*M* 5′AMP to the reaction mixture provides a measure of the total phosphorylase activity present in the sample. The extent of phosphorylase activation was expressed as the ratio of the activity measured in the absence of 5′AMP to that measured with 5′AMP. The specific activity of phosphorylase measured at 30°C was expressed as micromole of G-1-P per minute per gram weight of frozen tissue.

cAMP-Dependent protein kinase activity measurements. The extent of activation of cAMP-dependent protein kinase in each strip was assessed by calculating the ratio of kinase activity measured in the absence of cAMP to the activity measured with 2 μM cAMP. This ratio, as initially described by Corbin *et al.* (23–25), is an index of the relative amount of free catalytic subunit to the holoenzyme form of protein kinase in the tissue studied and reflects the extent to which the enzyme is activated *in vivo.* Previous studies in our laboratories showed that bovine coronary artery contains both type I and type II protein kinases (18). Accordingly, assays were performed with extracts prepared in the absence of added NaCl to detect the extent of activation of the type I isozyme, and in the presence of 0.5 *M* salt to detect the extent of activation of the type II isozyme (17, 18, 24, 26).

One half of each frozen strip (n = 50, 14 hearts) was homogenized (4°C) in 20 vol of 10 m*M* KH_2PO_4 (pH 6.8), 10 m*M* EDTA, 0.5 m*M* 1-methyl-3-isobutylxanthine, and 0.5 m*M* 1,4-dithiothreitol, whereas the second half of the strip was homogenized in the

same solution containing 0.5 *M* NaCl. The homogenates were centrifuged and the resulting supernatants were assayed for protein kinase activity (30°C) in either the presence or absence of 2 μM cAMP (18, 26). Activity was expressed as picomole ^{32}P incorporated into histone per minute per milligram of frozen tissue.

Statistical analysis. Data are expressed as means ± SEM. The data were analyzed using the one-way analysis of variance test and the Student Newman–Keuls multiple range test. A probability of $P < 0.05$ was taken as indicative of statistical significance.

Results. Two series of experiments were performed with strips of bovine coronary artery. Temporal relations between active isometric force and changes in phosphorylase activity were studied in the first series of experiments, whereas temporal relations between force and activity of cAMP-dependent protein kinase isozymes were studied in the second series.

Coronary arterial strips challenged with 30 m*M* KCl resulted in a time-dependent increase in isometric force (Figs. 1 and 2). Maximal force (109 ± mN, $n = 87$, 27 hearts)

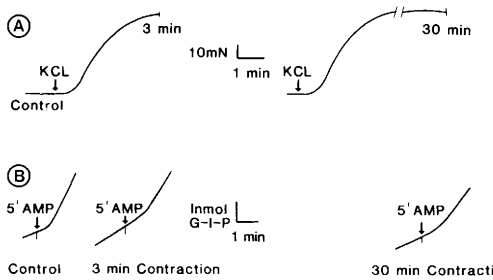

FIG. 1. Representative tracings are shown for isometric force (A) and phosphorylase activity (B) of a control strip, and for strips frozen after 3 and 30 min of contraction in the presence of 30 m*M* KCl. The vertical deflections in the fluorescence records correspond to addition of 5'AMP (2 m*M*, final concentration) to the phosphorylase reaction mixture. As described under Materials and Methods, the ratio of the rate change in fluorescence seen in the absence of 5'AMP (phosphorylase *a* activity) to the rate change in the presence of 5'AMP (total phosphorylase activity) is an index of phosphorylase activation (activity ratio). The tracings show that phosphorylase *a* activity and the consequent activity ratio increase as force increases (3 min) and then declines to a lower value even though maximal isometric force is maintained (30 min).

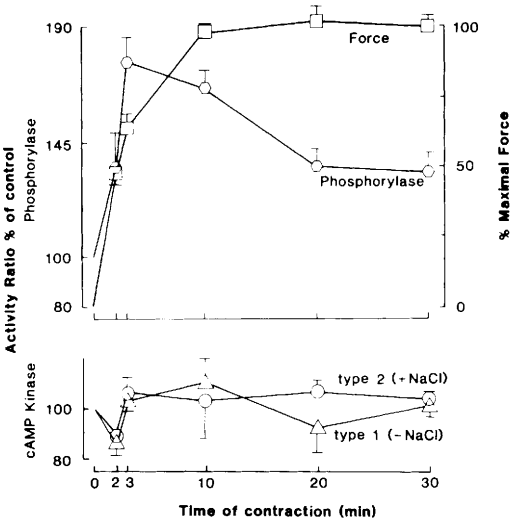

FIG. 2. The time course for development of isometric force and activation of phosphorylase during exposure to KCl is shown in the top panel whereas activities for type I and type II isozymes of cAMP-dependent protein kinase are shown in the bottom panel. Isometric force is expressed as a percentage of maximal isometric force attained (109 ± 4 mN) and activity ratios are expressed as percentage of control with respect to phosphorylase (0.18 ± 0.01, $n = 18$), and the type I (0.23 ± 0.01, $n = 25$) and type II (0.45 ± 0.01, $n = 25$) isozymes of protein kinase. Note that phosphorylase is apparently maximally activated before maximal isometric force is attained and that enzymic activity subsequently declines while force is maintained. In contrast, no change in kinase activities is evident during the contraction.

was attained after 10 min of exposure to KCl and was essentially unchanged during the remainder of the 30-min contracture.

In contrast to the monophasic development of force, a biphasic increase in the activation of phosphorylase occurred during the KCl-induced contractures (Figs. 1 and 2). Moreover, maximal activation of phosphorylase apparently occurred before maximal isometric force was attained. Thus, the greatest increase (175% of control) in phosphorylase activity (−5'AMP/+5'AMP) occurred after 3 min of exposure to KCl, whereas force had reached only about 65% of the maximal force attained. Subsequently, however, the extent of phosphorylase activation, though remaining above control, declined to a lower value (135% of control) which was maintained throughout the remainder of the contractile response. This biphasic change in phosphorylase activ-

ity was entirely ascribable to changes in activity assessed in the absence of 5'AMP (Table 1). That is, total phosphorylase activity, reflected by values obtained in the presence of 5'AMP, was unchanged throughout the contractile response. Total phosphorylase activity in 67 preparations averaged 0.412 ± 0.027 μmol G-1-P/min/g frozen wt. In contrast, phosphorylase activity determined in the absence of added 5'AMP increased to a maximum after 3 min of exposure to KCl, and then declined to a value which was maintained above control.

In accord with previous studies from this (18, 26) and other laboratories (17, 23–25), activity ratios for cAMP-dependent protein kinase (kinase activity without cAMP/kinase activity with cAMP) were dependent upon the presence or absence of 0.5 *M* NaCl in the homogenization medium. However, kinase activity ratios determined from homogenates prepared in either the presence or absence of 0.5 *M* NaCl did not change with time during the contractile response to KCl (Table II).

The control mean ratio obtained in the presence of NaCl (0.453 ± 0.013) was significantly greater than the ratio obtained in the absence of NaCl (0.227 ± 0.010, $P < 0.05$). Since NaCl is known to dissociate the type I isozyme of cAMP-dependent protein kinase (17, 24, 25) the apparent increase in activity seen in coronary homogenates prepared in the presence of salt is most likely ascribable to salt-induced dissociation (activation) of the type I isozyme (18, 26). Thus, the salt-induced increase in activity ratio was not associated with any significant change in total protein kinase activity measured in the presence of 2 μ*M* cAMP. Instead, the increase in ratio reflected the increase in kinase activity measured in the absence of added cAMP in homogenates prepared with 0.5 *M* NaCl. Thus, total kinase activities (+cAMP) were similar in extracts prepared with (10.29 ± 0.78 pmol ^{32}P/min/mg frozen wt) and without NaCl (8.23 ± 0.54 pmol ^{32}P/min/mg frozen wt). In contrast, kinase activities measured in the absence of cAMP, largely reflecting dissociated or activated cAMP-dependent kinase, were significantly greater ($F < 0.05$) with NaCl (3.64 ± 0.23 pmol ^{32}P/min/mg frozen wt) than when NaCl was omitted (2.27 ± 0.20 pmol ^{32}P/min/mg frozen wt) from the homogenization medium. These findings are in accord with the view that assays performed with homogenates prepared without added NaCl permit assessment of the activity of the type I isozyme, whereas assays with NaCl homogenates dissociate the type I isozyme and permit assessment of the activity of the type II isozyme (17, 18, 23–26).

Discussion. The results of this study show that a biphasic increase in the phosphorylase activity ratio occurs when coronary arterial

TABLE I. CHANGES IN PHOSPHORYLASE ACTIVITY DURING CONTRACTION (+30 m*M* KCl) OF BOVINE CORONARY ARTERIAL STRIPS

Time of contraction (min)	*n*	Phosphorylase activity (μmol G-1-P/min/g)		Activity ratio −5'AMP +5'AMP
		−5'AMP	+5'AMP	
0 (Control)	18	0.073 ± 0.006	0.412 ± 0.027	0.178 ± 0.007
2	7	0.103 ± 0.018[a]	0.430 ± 0.61	0.241 ± 0.013[a]
3	7	0.122 ± 0.013[a]	0.385 ± 0.026	0.314 ± 0.018[a,b]
10	7	0.114 ± 0.012[a]	0.391 ± 0.044	0.296 ± 0.013[a]
20	5	0.097 ± 0.016[a]	0.402 ± 0.041	0.241 ± 0.013[a]
30	10	0.104 ± 0.012[a]	0.422 ± 0.042	0.244 ± 0.015[a]

Note. Values are means ± SEM for duplicate determinations on the number (*n*) of strips indicated. Phosphorylase activity was determined as described under Materials and Methods. Control strips, incubated in the absence of KCl, were included for each period of contraction: since no significant difference was found among the control strips studied at the different time points all controls were grouped together.

[a] $p < 0.05$ compared to control.

[b] $p < 0.05$ compared to control and each time point.

TABLE II. CHANGES IN THE ACTIVITY OF cAMP-DEPENDENT PROTEIN KINASES DURING CONTRACTION (+30 m*M* KCl) OF BOVINE CORONARY ARTERIAL STRIPS

Time of contraction	*n*	No salt homogenates (type I activity) pmol ^{32}P/min/mg		Activity ratio −cAMP +cAMP	0.5 *M* NaCl Homogenates (type II activity) pmol ^{32}P/min/mg		Activity ratio −cAMP +cAMP
		−cAMP	+cAMP		−cAMP	+cAMP	
0 (Control)	25	2.27 ± 0.20	10.29 ± 0.78	0.227 ± 0.010	3.64 ± 0.23	8.23 ± 0.54	0.453 ± 0.013
2	4	2.18 ± 0.49	12.23 ± 2.82	0.184 ± 0.009	3.89 ± 0.55	8.61 ± 0.86	0.432 ± 0.029
3	3	2.50 ± 0.44	9.44 ± 1.14	0.235 ± 0.031	3.40 ± 0.22	7.40 ± 0.49	0.462 ± 0.021
10	7	1.83 ± 0.32	7.73 ± 1.34	0.235 ± 0.016	3.57 ± 0.63	8.24 ± 1.36	0.432 ± 0.024
20	8	1.65 ± 0.29	8.37 ± 1.51	0.196 ± 0.011	3.43 ± 0.44	7.49 ± 0.92	0.455 ± 0.016
30	3	2.72 ± 0.46	8.85 ± 2.65	0.261 ± 0.009	3.93 ± 0.14	8.15 ± 0.62	0.492 ± 0.030

Note. Values are means ± SEM for duplicate determinations on the number (*n*) of strips indicated. Frozen strips were divided and half of each strip was homogenized in the presence of 0.5 *M* NaCl (type II kinase), whereas the second half of the strip was homogenized in the absence of NaCl (type I kinase) and each preparation was assayed for kinase activity with and without 2 μM cAMP (see Materials and Methods). Control strips, incubated in the absence of KCl, were included for each period of contraction: since no significant difference was found among the control strips studied at the different time points all controls were grouped together. As described in the text, all kinase activities were higher in the presence of 0.5 *M* NaCl reflecting salt-induced activation of the type I isozyme. However, no significant difference in kinase activity was found between the controls and any of the contracted coronary strips assayed in either the presence or absence of 0.5 *M* NaCl.

smooth muscle is contracted with KCl (Figs. 1 and 2). That is, the phosphorylase activity ratio increases sharply immediately after stimulation with KCl, reaches an apparent maximum within 3 min of stimulation, and then, though remaining above control, declines to a lower level even though maximal isometric force is maintained. It is particularly noteworthy that the increase in phosphorylase activity ratio was entirely due to an increase in enzymic activity measured in the absence of 5'AMP (Table I). Thus, the increase in phosphorylase activity ratio obtained in these studies reflects formation of phosphorylase *a*. Recent studies of tracheal smooth muscle contracted with carbacol also show that activation of phosphorylase follows a biphasic time course (27).

The present findings, however, also show that no change in the activity ratios for cAMP-dependent protein kinases is manifest during the contractile response (Fig. 2, Table II). These results suggest that activation of phosphorylase during KCl-induced contraction of coronary arterial smooth muscle does not depend on the activation of cAMP-dependent protein kinases. Previous studies in our laboratories showed that bovine arterial smooth muscle contains both type I and type II isozymes of cAMP-dependent protein kinase (18). Moreover, determination of the protein kinase activity ratio showed that β-adrenergic relaxation of coronary strips contracted with KCl was highly correlated to activation of the type II isozyme. This specific activation was not attributable to spurious activation of the enzyme during homogenization of the arterial tissue, and was not manifest during β-adrenergic blockade with propranolol. The same analytical procedures were employed in the present study and showed that no activation of either the type I (no salt homogenization) or the type II isozyme (homogenization with 0.5 *M* NaCl) occurred during KCl-induced contraction (Table II). If the type I isozyme had been activated, then the kinase activity ratio determined in extracts prepared without 0.5 *M* NaCl would have increased (17, 23–25). Homogenization of tissues which contain both isozymes in 0.5 *M* NaCl is known to result in dissociation of the type I isozyme so that the basal activity ratio is expectedly elevated (17, 18, 23–26). If the type II isozyme had been activated during the contractile response to KCl, then the kinase activity ratio determined in homogenates prepared with 0.5 *M* NaCl would have increased progressively. Since the kinase ratios determined in either the presence or absence of 0.5 *M* NaCl did not increase, the biphasic increase in the activity ratio for phosphorylase which oc-

curred during KCl-induced contraction was not mediated by activation of either the type I or type II isozyme of cAMP-dependent protein kinase.

Recent work in our laboratories (9, 19) has indicated that the time course of vascular energy metabolism during a KCl contraction is also biphasic. Similarly, phosphorylation of the regulatory myosin light chains, like phosphorylation of phosphorylase, has been reported to be also biphasic (20, 27–30). Moreover, although fatty acids are likely to be the major oxidative substrate in VSM under resting conditions (31), we have recently shown that glycogen breakdown is likely to be the major substrate for the increase in oxygen consumption observed in a KCl-induced contraction (32).

The tight temporal coupling found between activation of phosphorylase and development of isometric force suggests that these events may be functionally coordinated (Fig. 2). Although the mechanism(s) by which this coordinated activity is achieved is unknown, it is unlikely to involve activation of cAMP-dependent protein kinases. Conceivably, such coordination may be linked to the fact that stimulation of phosphorylase kinase and initiation of contraction are both Ca^{2+}-dependent processes (5, 10, 11). Within this framework, biphasic activation of phosphorylase may reflect transient changes in the intracellular concentration of Ca^{2+} during the contractile response (33). On the other hand, as the state of protein phosphorylation *in vivo* may also be regulated by phosphatase activity, the possibility that phosphatases participate in coordinating arterial metabolism (via modulation of phosphorylase activity) and contractility (via modulation of the phosphorylation of myosin light chains) is also attractive (7, 8, 15). Since vascular contractility is critically dependent on continuous metabolic input (34), further elucidation of these mechanisms of coordination may be of considerable significance to our understanding of vascular myopathy.

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