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PROTEIN KINASE C ACTIVITY AND REACTIVITY TO PHORBOL ESTER IN VASCULAR SMOOTH MUSCLE FROM SPONTANEOUSLY HYPERTENSIVE RATS (SHR) AND NORMOTENSIVE WISTAR KYOTO RATS (WKY)

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Protein kinase C (PKC) activity in aortic and renal arterial smooth muscle from SHR (20-23 wk male; mean arterial pressure = 178 mm Hg) and WKY (age/sex matched; mean arterial pressure = 126 mm Hg) was quantitated. Activity was greatest in the particulate fractions relative to the soluble fractions in all sources. The only difference between SHR and WKY was in the soluble fraction from SHR renal arteries, which had 2 fold more activity (255 pmol/mg/min) when compared with WKY (136 pmol/mg/min). This difference was not apparently related to force modulation, since the magnitude of isometric force development in renal arteries in response to phorbol 12, 13-dibutyrate was not different between SHR and WKY. The magnitude of force developed in response to phorbol 12, 13-dibutyrate and PKC activity in the particulate fraction was greatest in aorta vs. renal arteries in both WKY and SHR. These results suggest that regional vascular differences in the amount of PKC activity may exist which are not apparently related to a disease state (i.e., hypertension). These differences may be related to differential sensitivity to phorbol ester-mediated contractions in isolated smooth muscle.

There is now ample evidence which suggests that protein kinase C plays an important role in maintaining isometric force in vascular smooth muscle (see 1 for review). Evidence supporting this hypothesis comes from studies which demonstrate sustained diacylglycerol formation by contractile agonists in intact smooth muscle cells or tissue (2,3), the ability of phorbol esters like phorbol dibutyrate and 12-0-tetradecanoylphorbol-1B-acetate (which substitute for diacylglycerol as activators of protein kinase C) to produce slow, substained contractions in various vascular smooth muscle preparations (4,5) in the absence of myosin light chain phosphorylation (6), and from experiments that have identified a putative unique phosphoprotein substrate (or substrates) in vascular or tracheal smooth muscle (7,8). Although the subcellular location of protein kinase C activity has been postulated to be in a particulate fraction (1), comparative studies quantitating soluble and particulate protein kinase C activity in vascular smooth muscle from different vascular beds are lacking.

There is also evidence which suggests that protein kinase C activity may be altered in a disease such as hypertension. In a prior study, age-related increases in

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systolic blood pressure in SHR were accompanied by parallel increases in protein kinase C activity in platelets (9); this increase in protein kinase C activity was interpreted as possibly being related to altered contractility in vascular smooth muscle that occurs in SHR, although no data with isolated vascular smooth muscle were presented.

Accordingly, the purposes of the present study were to 1) Quantitate the amount of protein kinase C activity in the two major subcellular fractions (soluble, particulate) from two sources (aortic, renal arterial) of vascular smooth muscle 2) Determine if there are differences between hypertensive (SHR) and age/sex-matched normotensive (WKY) rats 3) Examine the functional significance of these biochemical comparisons by quantitating the potency and efficacy of a protein kinase C activator (phorbol dibutyrate) on isometric force development in intact aortic and renal arterial smooth muscle.

Materials and Methods

Animals: Twenty to twenty-three week old SHR and WKY male rats were obtained from Charles River (Wilmington, MA). Mean arterial blood pressures, obtained via catheterization of the carotid artery, were kindly provided by Dr. Alan Buchholz of the Dept. of Pharmacology, Sterling-Winthrop Research Institute. Average pressure for the SHR group was 178± 6 mm Hg and for the WKY group was 126±3 mm Hg. To obtain aortic or renal arterial smooth muscle, animals were anesthetized with CO_2 prior to cervical dislocation. Arteries were removed from the animals and cleaned of connective tissue within 5-8 minutes after death.

Protein Kinase C Activity in the Soluble and Particulate Fractions: Protein kinase C activity was determined by slight modification of previously described procedures (9-11). Arterial strips were quickly homogenized, on ice, in 10 vols. of 20 mM Tris-HCl pH 7.4, 0.3 M sucrose, 2 mM EGTA, 5 mM EDTA, 5 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. Single aortae, weighing approximately 40 mg, were processed individually while three renal arteries (10 mg each) were pooled for each renal arterial preparation. Homogenates were centrifuged at 100,000 x g for 60 min. at 4° C; the supernatant fraction from this centrifugation represented the soluble fraction and was immediately assayed for protein kinase C activity. Pellets were resuspended in 250 µl of the original homogenization buffer supplemented with 0.1% Triton X-100, allowed to stand on ice for 30 min, and centrifuged at 30,000 x g for 20 min. The supernatant fraction of this centrifugation represented the particulate fraction. Pilot experiments revealed that no protein kinase C activity was present in the re-extracted pellet of this second centrifugation, and that 0.1% Triton-X 100 did not affect protein kinase C activity when added directly to the initial soluble fraction.

Protein kinase C activity in these fractions was quantitated at 30° C in reaction mixtures (70 µl) containing 20 mM Tris-HCl pH 7.4, 10 mM magnesium acetate, 200 µg/ml histone (Type IIIs - Sigma Chemial Co., St. Louis, Mo.), 100 µM ATP (containing 25 µCi/ml of γ ^{32}P-ATP, New England Nuclear) and 20 µl of either fraction. Basal kinase activity was determined in the presence of 5 mM EGTA while Ca^{2+} - phospholipid stimulated activity was determined in the presence of 1 mM $CaCl_2$ plus lipid micelles composed of 20 µg/ml phosphatidylserine plus 2 µg/ml diolein. Reaction mixtures (without the enzyme) were pre-incubated for 4 min. at 30°C; reactions were initiated by the addition of either the soluble or particulate fraction. Assays were terminated after 1.5 min. (soluble) or 3 min. (particulate) by spotting onto filter paper discs and immediate immersion into ice-cold 10% trichloroacetic acid/4% sodium pyrophosphate (10). Preliminary experiments showed that reactions for both fractions were linear over these respective time intervals. Duplicate samples were assayed for each sample; blanks contained

20 µl of extraction buffer only. Results are expressed as pmol ^{32}P incorporated into histone/mg protein/min. Protein content of both fractions was determined by the method of Bradford (12). Statistical analysis of all data was accomplished using the Student-Neuman-Kuels multiple comparisons test. A P value of less than 0.05 was used as the criterion for significance.

Intact Muscle Studies:

Arteries were placed in a modified Krebs solution of the following composition (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 1.6; $MgCl_2$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 24.9; dextrose, 11.1; $CaNa_2EDTA$, 0.026 (pH = 7.3-7.4). The vessels were cleaned of adjoining fatty and connective tisue, cut into rings 3-4 mm long and placed on ringholders via two stainless steel wires (40 gauge) threaded through the lumen. One wire was fastened to a holder which was connected to a Grass FT03 force displacement transducer; the other was attached to a fixed holder. The assembly was then immersed in a 10 ml jacketed organ bath filled with modified Krebs solution maintained at 37° C and bubbled with 95% O_2 and 5% CO_2. Blood vessels were placed under an optimal passive tension (0.5g for renal arteries, 2.0 g for aortae) and were allowed to equilibrate for 90 minutes, during which time the modified Krebs solution was changed every 15 - 20 min. After equilibration, a cumulative concentration response curve to phenylephrine was generated. Following a 60 min wash-out period, a cumulative concentration-response curve to phorbol 12, 13-dibutyrate (Sigma Chemical Co.) was generated.

Results and Discussion

Protein kinase C activity in the particulate fractions from both aortic and renal arterial smooth muscle was markedly higher (4-6 fold) than in the soluble fraction (Fig 1). Significant differences in activity were evident when the vascular

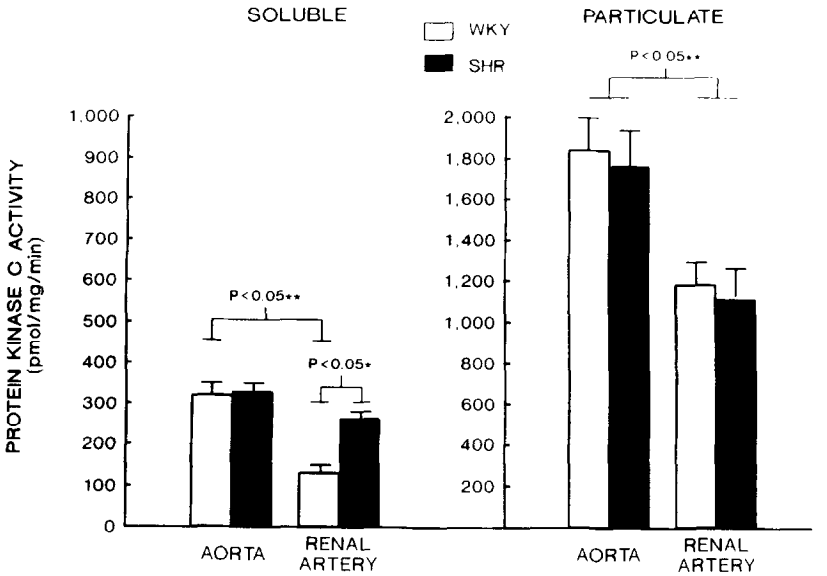

Fig. 1 Protein kinase C activity in soluble and particulate fractions from aortic or renal arterial smooth muscle isolated from SHR or age/sex-matched WKY, N = 10 preparations per determination (1 aortae or 3 renal arteries per preparation).

*P < 0.05; Comparison of WKY vs SHR

**P < 0.05; Comparison of aorta vs renal artery

smooth muscle sources were compared. For example, there was greater protein kinase C activity in both the soluble and particulate fractions from aortae when compared with renal arteries in normotensive animals. Similarly, protein kinase C activity from particulate fractions of aortae from SHR was greater when compared with similar fractions from SHR renal arteries. The only significant difference possibly related to hypertension was observed in the soluble fraction from SHR renal arteries, which had two-fold as much protein kinase C activity as found in their normotensive counterparts. No differences related to hypertension were evident in comparison to particulate fractions.

In intact vascular smooth muscle (Fig 2), the sensitivity to phorbol 12,13-dibutyrate was slightly (approximately two-fold) greater with both aortic and renal arterial smooth muscle isolated from SHR (EC_{50} aorta = 25 nM; EC_{50} renal artery = 50 nM) when compared with WKY blood vessels (EC_{50} aorta = 41 nM; EC_{50} renal artery = 100 nM). However, the magnitude of the contractile response to phorbol dibutyrate was not different between WKY vs. SHR when both sources were compared. A difference might be expected if the magnitude in force development was related to biochemical differences in protein kinase C activity (Fig 1). These data suggest that the two-fold greater protein kinase C activity present in the soluble fraction from SHR renal arteries is not relevant to an effect upon contractile function, since no difference between SHR and WKY renal arteries in the magnitude of the phorbol dibutyrate contraction was evident. While there was an enhanced sensitivity to phorbol dibutyrate in renal arteries from SHR, a similar increase in sensitivity in SHR aortae was evident. There were no differences in soluble or particulate protein kinase C activity in SHR vs WKY aortae. Further studies examining phospholipid and Ca^{2+} sensitivity of protein kinase C activity from SHR and WKY may further elucidate whether this increased sensitivity is related to protein kinase C or post kinase-mediated events. It is

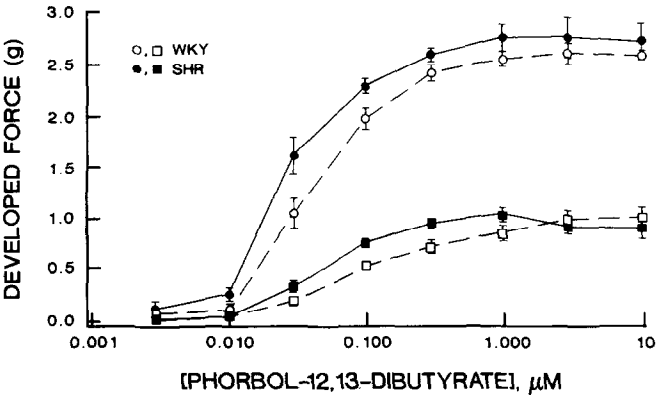

Fig 2. Phorbol ester - mediated isometric force development in intact aortic (circles) or renal arterial (squares) smooth muscle isolated from SHR or age/sex matched WKY, N = 16-28.

possible that enhanced protein kinase C activity in SHR renal arteries might be related to something other than direct modulation of force development.

The magnitude of isometric force development in response to phorbol dibutyrate was markedly greater when aortae were compared with renal arteries (Fig 2). Moreover, protein kinase C activity was substantially greater in the particulate fractions from the aortae relative to renal arteries (Fig 1). Correction for the maximum force generating capacity of renal arteries vs. aortae was made by quantitating the maximal force generation in response to another contractile agonist, phenylephrine. In this comparison, the maximum force (%) developed in response to phorbol ester/maximum force developed in response to phenylephrine was greater in the aortae (SHR 2.73g/1.64g = 167%; WKY 2.57g/1.83g = 140%) when compared with renal arteries (SHR 1.04g/0.85g = 122%; WKY 1.01g/0.88g = 115%). Maximum force generated by phorbol dibutyrate was significantly ($p < 0.05$) greater in both SHR and WKY aortae relative to the maximum force generated by phenylephrine; there were no significant differences in maximum force generated by phorbol dibutyrate relative to phenylephrine in renal arteries. While these data do not prove a link between particulate protein kinase C activity and force generation, they are consistent with the hypothesis that protein kinase C activity in the particulate fraction of vascular smooth muscle is important in regulating contraction in response to phorbol esters (1). Moreover, these data suggest that differences in protein kinase C activity exist in different vascular beds and are consistent with a previous study which demonstrated different contractile responses to phorbol dibutyrate with rabbit blood vessels from different vascular beds (13). Further examination of this hypothesis in various vascular beds from different species, as well as the correlation between particulate protein kinase C activity and phorbol ester - mediated isometric force generation, is underway in our laboratories.

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