Protein Kinase Inhibitors and Blood Pressure Control in Spontaneously Hypertensive Rats

R. Allan Buchholz, Ronald L. Dundore, Wayne R. Cumiskey, Alex L. Harris, and Paul J. Silver

Considerable evidence suggests that protein kinase C activation participates in the regulation of vascular smooth muscle tone. The objective of the current study was to examine the relations between inhibition of protein kinase C (PKC) and myosin light-chain kinase (MLCK) and vasorelaxation and blood pressure regulation in spontaneously hypertensive rats (SHR). Putative PKC inhibitors from two chemical classes, staurosporinelike (staurosporine and K252A) and isoquinolinesulfonamides (H7 and HA1004), were tested for their ability to 1) inhibit PKC and MLCK from SHR aorta, 2) relax isolated SHR aorta, and 3) lower blood pressure in conscious SHR. A rank order of potency for the inhibition of PKC and MLCK was established, with the staurosporinelike compounds (staurosporine PKC IC₅₀=54 nM) clearly more potent than the isoquinolinesulfonamides (H7 PKC IC₅₀=128 nM). The rank order of potency for inhibition of PKC was retained for inhibition of MLCK for all compounds. Staurosporine (EC₅₀=75 nM) and H7 (EC₅₀=2 µM) caused concentration-dependent relaxation of SHR aorta, but only staurosporine produced vasorelaxation at concentrations consistent with the inhibition of PKC or MLCK. Dose-dependent reductions in arterial pressure of SHR were demonstrated after intravenous injection of staurosporine and HA1004. A single intravenous injection of staurosporine (0.3 mg/kg) lowered blood pressure for more than 10 hours. Staurosporine also lowered blood pressure after oral administration. The depressor response to staurosporine was unaffected by sympathetic β-adrenergic blockade. In conclusion, the vasorelaxant and antihypertensive actions of staurosporine in SHR are consistent with the inhibition of PKC but could also be equally related to inhibition of MLCK. Not all PKC inhibitors produce vasorelaxation and lower blood pressure. Moreover, the lack of correlation between in vitro vasodilation and PKC or MLCK inhibition for the isoquinolinesulfonamide protein kinase inhibitors H7 and HA1004 suggests that these agents do not cause vasorelaxation in SHR by inhibition of these enzymes. (Hypertension 1991; 17:91-100)
myosin light chain phosphorylation. Moreover, selective inhibitors of PKC antagonize phorbol ester- and norepinephrine-induced vascular contractions (References 14, 15, and unpublished observations from our laboratory) and inhibit stretch-dependent vascular tone. 

Although PKC may participate in the maintenance of normal vascular tone, recent reports have also demonstrated that age-related increases in systolic blood pressure were paralleled by increases in PKC activity in the aorta and platelets of spontaneously hypertensive rats (SHR). Others have reported an increased sensitivity in the contractile response to phorbol esters in the isolated aorta and renal artery of SHR, although PKC activity was found to be elevated only in the soluble fraction of the renal artery. PKC activity in erythrocytes of patients with essential, but not renal, hypertension was also found to be significantly elevated. Thus, evidence exists that suggests heightened PKC activity may contribute to the elevation of vascular tone in hypertension.

Protein kinase inhibitors from two chemical classes, staurosporinelike and isoquinolinesulfonamides, have been reported to be relatively selective for PKC inhibition, although inhibition of other kinases (such as MLCK) is also apparent. Agents such as these are potentially useful in delineating the role of PKC in the regulation of vascular smooth muscle tone in normal and disease states (e.g., hypertension). However, no studies to date have clearly established a relation between the ability of these types of agents to inhibit PKC or MLCK and their ability to relax vascular smooth muscle and lower arterial blood pressure. Therefore, the objective of the present study was to determine the relation between PKC and MLCK inhibition and vasorelaxation and blood pressure regulation using putative PKC inhibitors of varied potency in biochemical, vascular tissue and in vivo models. Staurosporinelike and isoquinolinesulfonamide protein kinase inhibitors were tested for their ability to inhibit purified PKC and MLCK and their ability to relax vascular smooth muscle and lower arterial pressure of conscious SHR.

Methods

Determination of Protein Kinase C Activity

PKC was purified from male, adult whole rat (Sprague-Dawley, Charles River, Wilmington, Mass.) brain, as described previously. Isolation of the soluble and particulate fractions of PKC from SHR aorta (15–17 weeks old, Charles River) was performed, as detailed elsewhere. PKC activity was determined, as described by Silver et al., by using a slight modification of a previously described procedure. PKC activity at each concentration of kinase inhibitor was quantitated at 30°C in reaction mixtures (70 μl) containing: 20 mM Tris-HCl buffer (pH 7.4), 10 mM Mg<sup>2+</sup>-acetate, 200 μg/ml histone (Type IIIa, Sigma Chemical Co., St. Louis, Mo.); 10 μM ATP (containing 25 μCi/ml of γ<sup>32</sup>P-ATP, New England Nuclear, Boston, Mass.) for purified PKC or 100 μM ATP for the soluble and particulate fractions from SHR aorta; 20 μl purified PKC or 20 μl of the soluble or particulate fraction from SHR aorta; 7 μl kinase inhibitor. After a preincubation of the reaction mixture, without enzyme, for 4 minutes, the reactions were initiated by the addition of purified enzyme or extract. Assays were terminated after 1.5 minutes (soluble fraction) or 3 minutes (purified PKC, particulate fraction) by spotting onto filter paper discs and immediate immersion into ice-cold 10% trichloroacetic acid/4% sodium pyrophosphate. Reactions were linear for these time intervals. Results are expressed as picomoles phosphorus-32 incorporated into histone per milligram protein per minute. Protein content was determined by the method of Bradford. Basal kinase activity was determined in the presence of 10 mM EGTA. K<sub>v</sub> values for staurosporine and H7 against purified PKC were quantitated with respect to ATP concentration using multiple concentrations of ATP and the inhibitors.

Determination of Myosin Light-Chain Kinase Activity

MLCK was purified from bovine tracheal smooth muscle by minor modification of the method described by Hathaway et al. for bovine aortic smooth muscle. MLCK was also isolated from SHR aorta. Aortas were obtained as described previously. Individual tissues were minced and homogenized, on ice, in 80 vol of 20 mM MOPS buffer, pH 7.0, 1 mM EDTA, 5 mM dithiothreitol (DTT), 0.1% (wt/vol) Tween 80, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mg/1 each of leupeptin, soybean trypsin inhibitor, and pepstatin A. The supernatant fraction resulting from a 60-minute centrifugation at 100,000g at 4°C contained approximately 90% of the soluble MLCK activity. MLCK activity was quantitated at 30°C in reaction mixtures (70 μl) consisting of 20 mM MOPS buffer, pH 7.0, 1 mM DTT, 0.1% DTT, 0.1% (wt/vol) Tween 80, 20 μl purified MLCK or soluble fraction from SHR aorta and 7 μl of various concentrations of a kinase inhibitor. Reaction mixtures (without light chains) were preincubated for 15 minutes; reactions were initiated by the addition of 30 μM purified turkey gizzard 20 kDa light chains (Ocean Biologics, Edmonds, Wash.), 1 mM calmodulin, approximately 1 mM free calcium, 10 mM Mg<sup>2+</sup>-acetate and 1 mM ATP (containing 50 μCi/ml γ<sup>32</sup>P-ATP). Assays were terminated after 5 minutes and MLCK activity determined using the same filter paper procedure described for PKC activity. Basal kinase activity was determined in the presence of 10 mM EGTA. K<sub>v</sub> values for staurosporine and H7 against purified MLCK were quantitated with respect to ATP concentration using multiple concentrations of ATP and the inhibitors.

Intact Vascular Smooth Muscle Studies

Aortas excised from 15–17-week-old SHR were placed in a modified Krebs solution with the follow-
were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and arterial and venous cannulas were placed in the abdominal aorta and vena cava through the femoral artery and vein, respectively. The cannulas were tunneled subcutaneously, exteriorized at the nape of the neck, flushed with a solution of heparinized saline (200 units/ml) and sealed with a stainless steel plug. The wounds were sutured and a broad-spectrum antibiotic suspension (0.2 ml, Combiotic, Pfizer, Groton, Conn.) was administered intramuscularly. The rats were allowed at least 2 days recovery before testing.

Conscious, instrumented SHR were used to examine: 1) the relative potency of several kinase inhibitors for lowering mean arterial pressure (MAP) in vivo after intravenous administration; 2) the duration of action of a selected kinase inhibitor (staurosporine) for lowering MAP after a single intravenous administration; 3) the ability of an orally administered kinase inhibitor (staurosporine) to lower MAP; and 4) the contribution of sympathetic β-adrenergic activity to the hemodynamic mechanisms potentially contributing to the staurosporine-induced reduction in MAP. The rats were placed in individual Plexiglas testing boxes (28 cm × 18 cm × 30 cm). The arterial cannula was connected to a Statham pressure transducer (P23ID, Gould Inc., Cleveland, Ohio) positioned at the level of the heart. The pulsatile pressure signal was recorded continuously on a Grass polygraph (model 7D, Grass Instruments, Quincy, Mass.). Heart rate was calculated from the peak systolic pressure and the MAP was derived by electronically filtering the pulsatile pressure signal.

After a 1-hour adaptation period, three arterial pressure measurements were taken at 5-minute intervals. The mean of the three measurements represented the resting arterial pressure. The dose-response relation for lowering MAP was established by intravenous administration of the kinase inhibitors, a reference calcium channel blocker, nitrendipine, or vehicle over a 1-minute period. The kinase inhibitors and nitrendipine were given in a cumulative dose fashion, with a 10-minute interval between each dose. The vehicles were injected similarly at a constant volume/weight (ml/kg). The maximum reduction in arterial pressure that occurred during this interval was determined. The dose producing a 25% reduction in MAP (ED25) was calculated for each compound. The duration of the depressor action of one kinase inhibitor, staurosporine, was also compared with that of nitrendipine after a single intravenous injection of the approximate ED25 dose (300 µg/kg). Cardiovascular activity was recorded continuously for 10 hours after medication, with an additional 1 hour recording 25 hours after the initial injection. Pharmacological assessment of potential hemodynamic factors contributing to the fall in arterial pressure after intravenous administration of staurosporine was performed by pretreating additional groups of rats with the β-adrenergic antagonist nadolol28 (1 mg/kg i.v.) or saline (1 ml/kg i.v.) 5 minutes before injection of the ED25 dose of staurosporine. Cardiovascular activity was recorded continuously for 2 hours after injection of staurosporine. The ability of staurosporine to lower arterial pressure after oral administration was also examined in conscious SHR. After resting arterial pressure and heart rate were measured, as described above, staurosporine or vehicle was administered by gavage in a 1 ml/kg volume and cardiovascular activity was recorded continuously for 6 hours.

**Chemical Agents and Drugs**

The isoquinolinesulfonamide protein kinase inhibitors, H7 and HA1004, were purchased from Seikagaku America, Inc., St. Petersburg, Fla., and staurosporine and K252A were purchased from Kyowa Hakko USA, Inc., New York, and Calbiochem Co., La Jolla, Calif. Nitrendipine was synthesized at the Sterling Research Group, Rensselaer, N.Y. Nadolol was generously supplied by E.R. Squibb and Sons, Inc., Princeton, N.J. H7 and HA1004 were prepared in distilled water for all studies. Staurosporine and K252A were prepared in 1% dimethyl sulfoxide (DMSO) and distilled water for the biochemical studies and 30% DMSO and distilled water for the intact vascular smooth muscle studies. For the in vivo studies staurosporine, K252A, and nitrendipine were prepared in a 1:1:2 solution of PEG 400, ethanol, and saline. Nadolol was prepared in 0.9% normal saline. Intravenous injection volumes did not exceed 0.25
ml. All vehicles were determined to have no effect in the various test models.

Data Analysis

The IC_{50}, K_{v}, and EC_{50} values for the biochemical and intact muscle studies and the ED_{50} values for the in vivo dose–response study were determined as described by Tallarida and Murray.^{29} The data from the in vivo intravenous and oral studies were analyzed by a two-way analysis of variance with one repeated measure.^{30} Post-hoc analyses of significant overall effects were performed using the Newman-Keuls multiple comparisons test or an unpaired Student's t test, as appropriate. Differences were considered statistically significant at p<0.05. All values are presented as mean±SEM.

Results

Inhibition of Protein Kinase C Activity

All compounds tested exhibited a concentration-dependent inhibition of PKC activity (Figure 1, upper panel). As summarized in the table of IC_{50} values shown in the upper portion of this panel, a rank order of potency for inhibition of purified PKC was evident, with the staurosporinelike compounds clearly more potent than the isoquinolinesulfonamides. Staurosporine, the most potent inhibitor (IC_{50}=12 nM) was approximately 5,000 times more potent than H7 (IC_{50}=54 μM), and HA1004, the least potent inhibitor (data not shown) was approximately threefold to fivefold less potent than H7. A concentration-dependent inhibition of the soluble and particulate fractions of PKC, extracted from SHR aorta, was also found for staurosporine and H7. No difference in the potency for inhibition of either fraction was apparent for staurosporine or H7. However, there was a slight shift to the right in the inhibition curves generated for staurosporine and H7 against partially purified PKC from SHR aorta versus purified PKC. This is most likely due to the difference in the ATP concentrations of the reaction mixtures used to assay the different sources of PKC (see Methods).

Inhibition of Myosin Light-Chain Kinase Activity

All compounds tested exhibited a concentration-dependent inhibition of MLCK activity (Figure 1, lower panel). As shown in the table of IC_{50} values, the rank order of potency observed for the inhibition of PKC was retained for the inhibition of MLCK. Moreover, there was no difference between the inhibition of purified bovine tracheal smooth muscle MLCK and MLCK extracted from SHR aorta for any of the compounds tested.

Vasorelaxation Versus Kinase Inhibition

Shown in Figure 2 are the concentration–response curves for the inhibition of PKC and MLCK activity and vasorelaxation produced by staurosporine (upper panel) and H7 (lower panel). Staurosporine produced a concentration-dependent relaxation of phenylephrine-contracted SHR aorta, with an EC_{50} value of 75 nM. The vasorelaxation curve for staurosporine fell between and overlapped the concentration–response curves for both PKC and MLCK inhibition. H7 also caused a concentration-dependent relaxation of aortic smooth muscle (EC_{50}=2 μM) but was much less potent than staurosporine. In contrast to staurosporine, the vasorelaxation curve for H7 was clearly positioned to the left of the PKC and MLCK inhibition curves. HA1004 also exhibited a response pattern similar to H7 with regard to the relative positions of the vasorelaxation and kinase inhibition curves (data not shown) (i.e., the curve depicting vasorelaxation was to the left of the inhibition curves for PKC and MLCK). However, K252a failed to
Concentration-response curves comparing the ability of staurosporine (STAUR0) (upper panel) and H7 (lower panel) to inhibit protein kinase C (PKC) and myosin light-chain kinase (MLCK) activity and relax phenylephrine-contracted spontaneously hypertensive rat (SHR) aortic rings (SHR AO). Curves for inhibition of PKC and MLCK activity by staurosporine and H7 are the same as displayed in Figure 1, with 100% representing complete inhibition of enzyme activity, as determined in the presence of 10 μM EGTA. K_i values for inhibition of enzyme activity and the EC_{50} for relaxation of aortic smooth muscle are shown in the lower corner of each panel. K_i values were quantitated with respect to ATP concentration using multiple concentrations of ATP and the inhibitors, as described in Methods. EC_{50} values for vasorelaxation were determined in cumulative concentration-response studies using phenylephrine-contracted SHR aortas, with 100% relaxation representing maximal relaxation induced by papaverine, as described in Methods. Vehicles for each compound had no effect on PKC or MLCK activity or vasorelaxation (data not shown). Values are mean±SEM.

cause significant vasorelaxation (less than 50%) in concentrations up to 300 μM. The selectivity of each inhibitor could not be determined on the basis of the IC_{50} values because of the difference in the ATP concentrations used in the PKC and MLCK assays. Therefore, the K_i values (relative to ATP concentration) of staurosporine and H7 for PKC and MLCK were determined (shown in lower corner of each panel of Figure 2). Based on K_i values, both staurosporine and H7 were fourfold to fivefold more potent for the inhibition of PKC as compared with MLCK. The K_i values of staurosporine for PKC and MLCK were both less than the EC_{50} value for aortic smooth muscle relaxation. In contrast, the K_i values of H7 for PKC and MLCK were both greater than the EC_{50} value for vasorelaxation. These findings suggest that inhibition of PKC or MLCK may contribute to the vasorelaxant action of staurosporine but not H7.

Cardiovascular Effects of Kinase Inhibitors

The effects of cumulative intravenous injections of nitrendipine, staurosporine, K252A, H7, HA1004, and vehicle on the MAP of conscious SHR are displayed in Figure 3. Nitrendipine and staurosporine produced dose-dependent reductions in MAP with comparable potency, as indicated by similar ED_{25} values (table inset, Figure 3). However, staurosporine exhibited a much steeper dose-response relation for lowering MAP than nitrendipine. HA1004 was the only other compound that produced at least a 25% reduction in MAP. K252A caused only a slight reduction in MAP at the largest dose tested. Larger doses were not tested due to the limits of solubility for K252A. H7 failed to reduce MAP at lower doses and produced seizures on administration of a 6 mg/kg dose, the largest dose tested. Because staurosporine was the only compound that exhibited potent inhibition of PKC, vasorelaxation and in vivo depressor actions, it was selected for additional cardiovascular evaluation in conscious SHR.

Preliminary evidence suggested that the depressor response to intravenous staurosporine had a long duration of action. Therefore, the duration of the arterial pressure response to equihypotensive doses of staurosporine, nitrendipine, and vehicle were evaluated after single intravenous, bolus injections to chronically instrumented, conscious SHR. Nitrendipine (0.2 mg/kg) produced a maximum reduction in MAP during the first 5 minutes after drug administration (Figure 4). The fall in MAP produced by staurosporine (0.3 mg/kg) was more gradual, with the maximum reduction achieved 15 minutes after medication. Both staurosporine and nitrendipine lowered MAP significantly for the first hour. Although the maximum reduction in MAP of animals given staurosporine and nitrendipine was the same, MAP remained significantly lower 30 minutes after injection in SHR given staurosporine than those administered nitrendipine. MAP returned to control levels within 2 hours after injection of nitrendipine. In contrast, staurosporine reduced MAP significantly for the entire 10-hour recording period. There were no significant differences in MAP among the three groups 25 hours after medication. Both staurosporine and nitrendipine produced a significant tachycardia. The tachycardia associated with intravenous staurosporine persisted throughout the 10-hour recording period and was still evident 25 hours after injection. Figure 5 shows the arterial pressure and heart rate responses to intravenous administration of staurosporine (0.3 mg/kg) after β-adrenergic blockade with nadolol (1 mg/kg i.v.). Nadolol had no effect on the MAP response to staurosporine; the curves depicting the MAP response to staurosporine in the presence
and absence of nadolol were virtually identical. However, nadolol eliminated the tachycardia caused by staurosporine. Nadolol alone did not significantly affect MAP but did significantly decrease heart rate in rats subsequently treated with vehicle.

Oral administration of staurosporine (1.8 mg/kg) produced a gradual reduction in the MAP of conscious SHR (Figure 6). The maximum reduction in MAP occurred 6 hours after medication. A slight, but non-significant increase in heart rate was observed in both the vehicle- and staurosporine-treated rats. This effect was probably due to the handling associated with oral drug administration. The absence of the persistent tachycardia that was observed after intravenous injection of staurosporine is probably related to the very gradual fall in arterial pressure noted after oral administration of this agent.

**Discussion**

The purpose of the present study was to examine the relation between PKC inhibition, vasorelaxation, and ultimately, blood pressure regulation in conscious SHR. Putative PKC inhibitors from two chemical classes, staurosporinlike and isoquinolinesulfonamides, were tested for their ability to inhibit PKC and MLCK from different tissue sources, relax vascular smooth muscle, and lower blood pressure in SHR. All compounds tested demonstrated a concentration-dependent inhibition of PKC and MLCK from different tissue sources, relax vascular smooth muscle, and lower blood pressure in SHR. All compounds tested demonstrated a concentration-dependent inhibition of PKC and MLCK. Staurosporine and K252A were more potent inhibitors of PKC and MLCK than the isoquinolinesulfonamides, H7 and HA1004, a finding in agreement with previous studies.

There was no difference in the potency for inhibition of the soluble and particulate fractions of PKC from SHR aorta for either staurosporine or H7. Inhibition of purified MLCK and MLCK isolated from SHR aorta was also similar for all compounds tested. Staurosporine was more potent as a vasorelaxant in vitro than H7 and only staurosporine and HA1004 produced significant dose-dependent reductions in MAP after intravenous administration. A single intravenous injection of staurosporine produced a prolonged (more than 10 hours) reduction in MAP and elevation in heart rate in conscious SHR in comparison with an equi-hypotensive dose of the calcium channel blocker nitrendipine. Oral administration of staurosporine to SHR also lowered MAP but without the persistent tachycardia observed after intravenous injection.

Protein kinase C has been localized in cytosol and membrane fractions where both enzymes appear to be similar. Although PKC activity in the particulate fraction of SHR aorta is four to six times higher than that in the soluble fraction, we found that the potency for inhibition of each fraction was similar for both staurosporine and H7. However, the concentration-response curves for inhibition of the soluble and particulate fractions of PKC from SHR aorta were shifted to the right fourfold to fivefold for staurosporine and twofold to threefold for H7 when compared with the inhibition curves for purified PKC. Different ATP concentrations were used to assay PKC activity purified from rat brain (10 μM ATP) and isolated from SHR aorta (100 μM ATP) to obtain linear reactions for each tissue source. Thus, the most likely explanation for the rightward shift in the inhibition curves is due to the difference in the ATP concentrations since both agents inhibit PKC by competing for the ATP binding site on the catalytic domain, which is conserved across PKC isoforms. In contrast, the same ATP concentrations were used to assay MLCK activity purified from tracheal smooth muscle and isolated from SHR aorta, resulting in virtually identical inhibition curves for each tissue source for all inhibitors tested.

The isoquinolinesulfonamide H7, although referred to as a specific PKC inhibitor, and the staurosporinlike compounds appear to show little selectivity for the inhibition of other protein kinases (e.g., cyclic AMP (cAMP)-dependent protein kinase, cyclic GMP (cGMP)-dependent protein

**Figure 3.** Dose-response curves demonstrating the ability of staurosporine, K252A, H7, and HA1004, and the calcium-channel blocker nitrendipine to lower mean arterial pressure (MAP) in conscious spontaneously hypertensive rats (SHR). Changes in MAP were quantitated via recordings made from indwelling aortic cannulas after cumulative intravenous doses of each compound. Dose producing a 25% reduction in MAP, defined as the ED<sub>25</sub>, was calculated using linear regression analysis and is displayed in the table inset to the left of the curves. All values are expressed as mean±SEM from four to eight rats per compound. NA, not achieved.

**FIGURE 3.** Dose-response curves demonstrating the ability of staurosporine, K252A, H7, and HA1004, and the calcium-channel blocker nitrendipine to lower mean arterial pressure (MAP) in conscious spontaneously hypertensive rats (SHR). Changes in MAP were quantitated via recordings made from indwelling aortic cannulas after cumulative intravenous doses of each compound. Dose producing a 25% reduction in MAP, defined as the ED<sub>25</sub>, was calculated using linear regression analysis and is displayed in the table inset to the left of the curves. All values are expressed as mean±SEM from four to eight rats per compound. NA, not achieved.
FIGURE 4. Duration of mean arterial pressure (MAP) and heart rate (HR) responses after a single intravenous injection of equihypotensive dose of staurosporine or nitrendipine in conscious spontaneously hypertensive rats (SHR). Cardiovascular activity was monitored via indwelling aortic cannulas. Left panel: MAP and HR responses during first 60 minutes after injection. Middle panel: Responses from 2 to 10 hours after injection. Right panel: MAP and HR values 25 hours after injection. Note persistent reduction in MAP and elevation in HR throughout the first 10 hours of monitoring. Values are expressed as mean±SEM. *Indicates significant difference versus vehicle control group, p<0.05.

kinase, and tyrosine protein kinase. Based on their $K_v$ values, staurosporine and H7 in the present study exhibited only a fourfold to fivefold greater potency for PKC versus MLCK inhibition (Figure 2), a finding consistent with previous reports and indicative of the relatively low degree of specificity observed with these types of inhibitors. Thus, the rank order of potency for PKC inhibition (staurosporine > K252A > H7 > HA1004) found in the current study was not unexpectedly retained for MLCK inhibition. The failure to demonstrate specificity for PKC inhibition with these agents is not surprising given the high degree of homology between the ATP binding site and catalytic domain of PKC with the same domains of other protein kinases. Greater selectivity for PKC inhibition may come with inhibitors acting at the regulatory domain of this enzyme, as recently described for calphostin.

Staurosporine and H7 produced concentration-dependent relaxation of SHR aorta, but only staurosporine caused vasorelaxation at concentrations consistent with the inhibition of PKC activity (Figure 2). However, comparison of the $K_v$ values of staurosporine for PKC and MLCK does not permit attribution of its vasorelaxant action exclusively to inhibition of PKC since inhibition of MLCK may also be involved. In contrast, the vasorelaxation caused by H7 clearly occurred at concentrations below those required to inhibit PKC or MLCK. Thus, inhibition of these protein kinases does not appear to be the mechanism or mechanisms responsible for the vasorelaxation caused by H7. Although both staurosporine and H7 are known to inhibit cAMP-dependent and cGMP-dependent protein kinases, it is unlikely that these effects contribute to the vasorelaxant action of either compound since inhibition of these kinases would presumably result in contraction of vascular smooth muscle. HA1004 may relax vascular smooth muscle by affecting intracellular Ca$^{2+}$, but this does not appear to be the mechanism of action for H7. K252A (also known as SF2370) failed to relax phenylephrine-contracted SHR aorta as previously re-
motensive rats. As indicated previously, the depressor response caused by HA1004 was probably related to its antagonism of intracellular Ca++ rather than inhibition of PKC or MLCK. It is apparent from these data that not all PKC inhibitors, even those that relax vascular smooth muscle in vitro, produce antihypertensive effects in vivo.

Staurosporine was the only agent that demonstrated vasorelaxant and antihypertensive actions that were consistent with the inhibition of PKC or MLCK. Both intravenous and oral administration of staurosporine had a significant antihypertensive effect in conscious SHR. The duration of the depressor response caused by staurosporine after intravenous injection was five to 10 times that of the calcium channel blocker nitrendipine. A sustained depressor response to oral staurosporine was also observed. Elimination of sympathetic β-adrenergic drive to the myocardium with the β-adrenergic antagonist nadolol had no effect on the magnitude or character of the depressor response to staurosporine. Thus, changes in cardiac contractility, heart rate, and cardiac output mediated through this system did not appear to contribute to the staurosporine-induced fall in arterial pressure in conscious SHR. A direct action of staurosporine on the heart that might influence these hemodynamic functions was not examined. However, we have recently found that the depressor response to intravenous staurosporine in conscious, normotensive dogs is mediated by a decrease in total peripheral vascular resistance and no reduction in cardiac output, a finding consistent with the in vitro vasorelaxant actions of staurosporine. The relatively rapid and sustained reduction in arterial pressure after intravenous injection of staurosporine was accompanied by a persistent tachycardia. The tachycardia associated with intravenous injection of staurosporine appeared to be of baroreceptor reflex origin and was exclusively mediated by enhanced sympathetic discharge to the heart since it was eliminated by β-adrenergic blockade. In contrast, the fall in arterial pressure after oral staurosporine was very gradual, reaching its nadir at 6 hours, and was not associated with a persistent tachycardia. The gradual reduction in pressure caused by orally administered staurosporine may have allowed a rapid resetting of the arterial baroreceptor reflex, obviating the tachycardic response.

Compounds derived from K252A (SF2370) and structurally related to staurosporine have also been shown to produce a long-lasting antihypertensive effect in conscious SHR. These compounds inhibited PKC activity and superprecipitation of actomyosin, the latter suggesting inhibition of MLCK activity. The potency for inhibition of PKC by these compounds was threefold to 12-fold greater than that for inhibition of superprecipitation of actomyosin. However, the concentrations required to produce relaxation of guinea pig aorta with these K252a analogues (20–100 μM) were in excess of those needed to inhibit either PKC activity or superprecipitation of actomyosin. These findings are consistent with the inability of this compound to penetrate the cellular membrane. Additional complications involved in comparing biochemical kinase experiments with intact cellular models include major differences in assay conditions (i.e., phosphoprotein substrates, ATP concentration) in the biochemical assays relative to conditions in intact vascular smooth muscle cells.

Staurosporine and HA1004 dose-dependently lowered arterial pressure in conscious SHR after intravenous administration. Despite having a relaxant effect on SHR aortic smooth muscle, H7 failed to lower arterial pressure in conscious SHR but instead caused seizures at the highest dose tested (6 mg/kg i.v.). The mechanism mediating this effect is unknown. The failure to lower arterial pressure with K252A is consistent with the absence of a vasorelaxant effect in SHR aorta, although K252A (SF2370) analogues have been found to cause dose-dependent reductions in arterial pressure in anesthetized, normotensive rats. As indicated previously, the depressor response caused by HA1004 was probably related to its antagonism of intracellular Ca++ rather than inhibition of PKC or MLCK. It is apparent from these data that not all PKC inhibitors, even those that relax vascular smooth muscle in vitro, produce antihypertensive effects in vivo.

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Compounds derived from K252A (SF2370) and structurally related to staurosporine have also been shown to produce a long-lasting antihypertensive effect in conscious SHR. These compounds inhibited PKC activity and superprecipitation of actomyosin, the latter suggesting inhibition of MLCK activity. The potency for inhibition of PKC by these compounds was threefold to 12-fold greater than that for inhibition of superprecipitation of actomyosin. However, the concentrations required to produce relaxation of guinea pig aorta with these K252a analogues (20–100 μM) were in excess of those needed to inhibit either PKC activity or superprecipitation of actomyosin. These findings are consistent with the inability of this compound to penetrate the cellular membrane. Additional complications involved in comparing biochemical kinase experiments with intact cellular models include major differences in assay conditions (i.e., phosphoprotein substrates, ATP concentration) in the biochemical assays relative to conditions in intact vascular smooth muscle cells.

Staurosporine and HA1004 dose-dependently lowered arterial pressure in conscious SHR after intravenous administration. Despite having a relaxant effect on SHR aortic smooth muscle, H7 failed to lower arterial pressure in conscious SHR but instead caused seizures at the highest dose tested (6 mg/kg i.v.). The mechanism mediating this effect is unknown. The failure to lower arterial pressure with K252A is consistent with the absence of a vasorelaxant effect in SHR aorta, although K252A (SF2370) analogues have been found to cause dose-dependent reductions in arterial pressure in anesthetized, normotensive rats. As indicated previously, the depressor response caused by HA1004 was probably related to its antagonism of intracellular Ca++ rather than inhibition of PKC or MLCK. It is apparent from these data that not all PKC inhibitors, even those that relax vascular smooth muscle in vitro, produce antihypertensive effects in vivo.
with those of the present study and suggest that these agents may cause vasorelaxation by inhibition of MLCK or PKC. Thus, the use of currently available protein kinase inhibitors, such as those examined in the present study, to clearly delineate the role of PKC in the regulation of vascular smooth muscle tone in normotension and hypertension may be limited by their lack of specificity for kinase inhibition or other pharmacological actions.

In summary, putative PKC inhibitors from two chemical classes were shown to inhibit PKC and MLCK with a similar rank order of potency. The potency for inhibition of the soluble and particulate fractions of PKC and soluble MLCK isolated from SHR aorta were similar to the inhibition of purified PKC and MLCK. The vasorelaxant and antihypertensive actions of staurosporine are consistent with the inhibition of PKC but could be equally related to inhibition of MLCK. However, not all compounds that inhibit PKC or MLCK produced vasorelaxation and lowered arterial pressure in SHR. The vasorelaxation caused by the reportedly selective PKC inhibitor H7 does not appear to be related to inhibition of either PKC or MLCK. Evaluation of highly selective inhibitors acting on the regulatory domain of PKC will help clarify the role of this enzyme in regulating smooth muscle tone and as a potential novel therapeutic target for the treatment of hypertension.

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References

18. Takaori K, Itoh S, Kanayama Y, Takeda T: Protein kinase C activity in platelets from spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto rats (WKY). Biochem Biophys Res Commun 1986;141:769–773


42. Ruzycky AL, Morgan KG: Involvement of the protein kinase C system in calcium-force relationships in ferret aorta. *Br J Pharmacol* 1989;97:391-400


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R A Buchholz, R L Dundore, W R Cumiskey, A L Harris and P J Silver

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