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ROLE OF PROTEIN KINASE C IN THE REGULATION OF PUMPING ACTIVITY IN BOVINE MESENTERIC LYMPHATIC VESSELS

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ABSTRACT

We investigated the role of protein kinase C (PKC) in regulating the lymphatic myogenic response. Bovine mesenteric lymphatics were suspended in an organ bath with inflow and outflow ends cannulated. Input was provided from a reservoir filled with Krebs solution. The PKC activator phorbol 12-myristate 13-acetate (PMA) inhibited pumping significantly whether tested at a fixed pressure or as pressures were raised in 2 cm H_2O increments (50% inhibition achieved at 4.6 X 10^{-8}M). The inactive phorbol ester (4-α-PMA) had no effect. The specific PKC inhibitors calphostin (10^{-9} to 10^{-7}M) or chelerythrine (10^{-8} to 10^{-6} M) had no significant effect on pumping. However, chelerythrine (10^{-6}M) was capable of reversing the inhibitory effects of PMA (5 X 10^{-8}M). PKC activation is believed to inhibit nitric oxide (NO) production in some blood vessels, and previous work from our laboratory has demonstrated that NO is important in facilitating pumping activity in bovine lymphatics. We observed that sodium nitroprusside (sNP, 10^{-7}M) or L-arginine (10^{-4}M), reversed the depressor effects of PMA. These results suggest that PKC may not be involved in regulating the vessel's contractile response to pressure-induced stretch. However, the data with PMA suggest that these ducts contain PKC. PKC activation depresses lymphatic pumping and this effect may be mediated in part, by inhibition of NO.

Lymphatic vessels, like their blood vascular counterparts, display myogenic activity. Unlike blood vessels however, lymphatic contractions provide a major portion of the energy required for propulsion of plasma ultrafiltrate back to the bloodstream with valves directing the flow centrally. Perhaps the most important stimulus for contractile activity is stretch of the vessel wall as the lymphatic fills with fluid. Like blood vessels, the lymphatic myogenic response exists in the absence of neural or humoral influences (1). Additionally, endothelial cells do not appear to be involved in the resting myogenic response at least in postnodal bovine mesenteric lymphatics (2).

Since filtration and lymph flow are closely integrated, lymphatic myogenic activity plays an important role in the regulation of interstitial volume in many tissues and organs. Unfortunately, little information is available on the molecular mechanisms that regulate this activity. Assuming that some of the biochemical transduction pathways proposed for blood vessels (3) are also relevant to lymphatics, the regulation of lymphatic contractions is likely to be complex. One potentially important regulatory mechanism has emerged from a consideration of the pathophysiological impact of tissue injury and hemorrhage on lymphatic function.

With vascular damage, RBCs extravasate into the tissues. Under these conditions,

oxyhemoglobin can be released from the cells and enter draining lymph. Oxyhemoglobin depresses lymphatic pumping activity (4-7). Since oxyhemoglobin is known to inhibit NO, these studies suggest that the L-arginine pathway is involved in the regulation of lymphatic contractions. In support of this hypothesis, N^G-monomethyl-L-arginine (L-NMMA) and methylene blue also inhibit lymphatic pumping and this inhibition (and that of oxyhemoglobin) is reversible with the NO substrate L-arginine or with the NO donor, sodium nitroprusside (sNP) (8). These results imply that the basal production of NO facilitate lymphatic pumping and suggest a prominent role for NO in regulating the lymphatic myogenic response.

Since NO synthesis within lymphatics has physiological and pathophysiological implications, we have continued to investigate the mechanisms responsible for NO regulation. In blood vascular preparations, phorbol ester-induced activation of PKC impairs endothelial-dependent relaxation (9). PKC inhibits nitric oxide synthase in endothelium (10) and smooth muscle (11). In addition, PKC has also been proposed as one of the transduction pathways important in modulating blood vessel myogenic activity (3). The observation that NO is closely associated with PKC in blood vessels, suggests one potential NO regulatory mechanism that appears to warrant investigation in lymphatics. The purpose of this investigation was to test whether PKC modulates the lymphatic contractile response and whether PKC activity is associated with an effect on NO.

MATERIALS AND METHODS

Organ Bath Preparation

The collection and suspension of lymphatic vessels in an organ bath have been described elsewhere (8). Briefly, bovine lymphatic vessels were isolated from the mesentery within ten minutes of slaughter. Vessels 8-10cm in length were cannulated at each end with PE-240 tubing (Intramedic Clay Adams). Vessel wall integrity was ensured by distending the vessel with a solution of 0.001% Evan's blue dye (in Lactated Ringer's Injection U.S.P., Baxter Corporation) to a transmural pressure of 10-12cm H_2O. Leaky vessels were discarded. Intact vessels were placed into a water-jacketed organ bath circulated with oxygenated (95% O_2-5% CO_2) Krebs solution (in mM: 120 NaCl, 5.9 KCl, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 2.5 $CaCl_2$, 19.0 $NaHCO_3$, 5.5 D-glucose, BDH, Inc.). Temperature and pH of the circulated fluid were maintained at 38°C and at 7.4 (φ12 pH/ISE meter, Beckman Instruments Inc.), respectively. The lymph vessels were fed from a common reservoir (10 mL volume) and were arranged in parallel with respect to each other. The reservoir contained either Krebs solution alone or Krebs plus the drug to be tested. The fluid in the reservoir was replenished from a larger supply at a rate of 10 mL/minute. To provide a constant transmural pressure the surface of the fluid in the 10 mL reservoir was level with the outflow port from the lymph vessels. The height of this fluid level (in cm) above the surface of the Krebs solution in the bath (zero reference point) determined the transmural pressure. The organ baths that were used in this study were designed to take up to 8 vessels at a time. However, due to time constraints imposed at the Abattoir, the number of lymphatics was usually restricted to ~ 6. Of these, several may not pump after the application of a transmural pressure. Typically, 2 to 4 lymph vessels were used for each experiment.

Experimental Protocols

In one set of experiments, a fixed transmural pressure of 4 or 6 cm H_2O was applied to the lymph vessels. The experimental protocol consisted of a one hour control period during which time vehicle was presented to the vessels. The volume pumped was calculated from the weight of the fluid

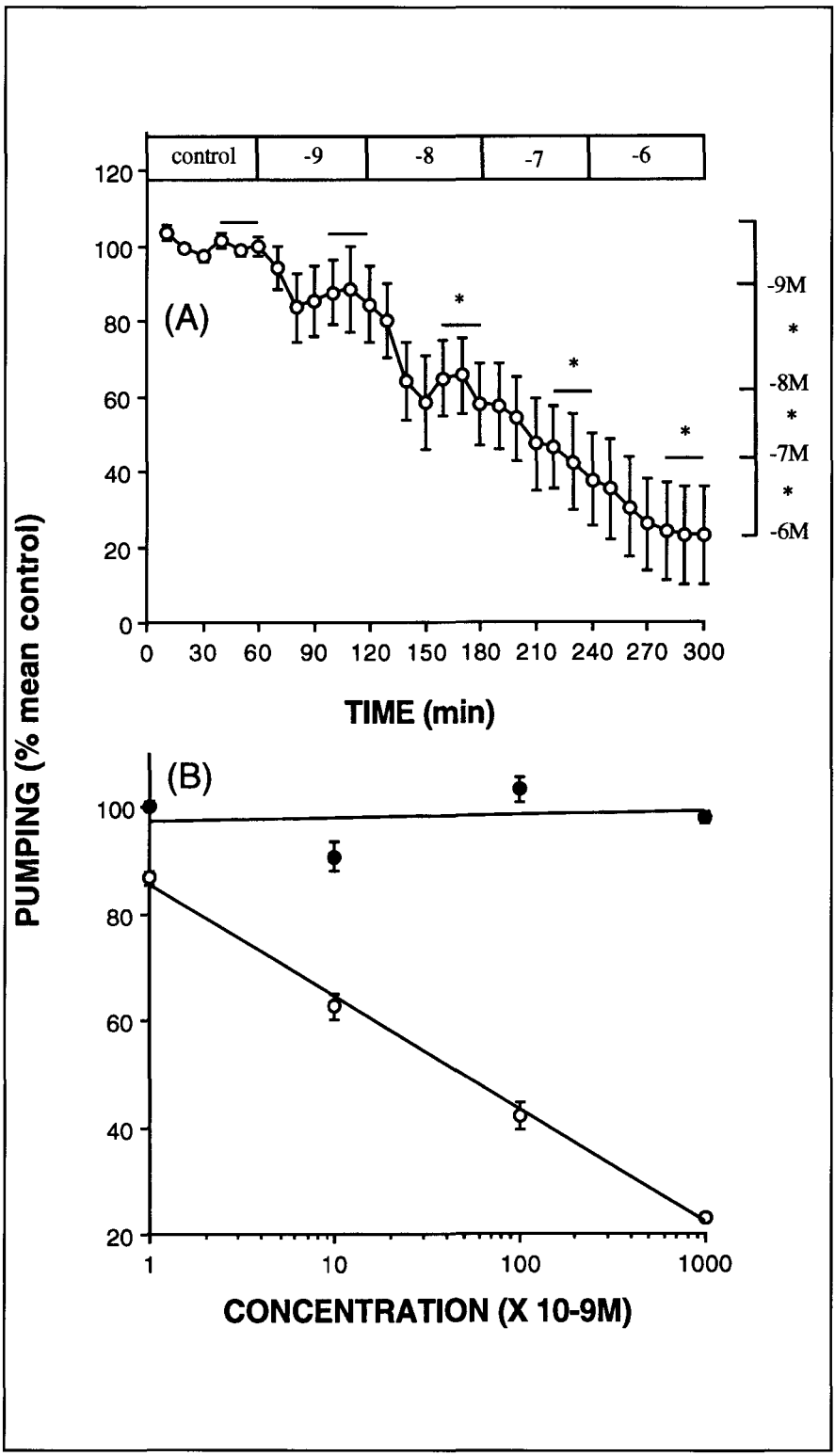

Fig. 1. Effects of PMA on lymphatic pumping. Following a 1 hour control period, PMA was added to the reservoir at concentrations between 10^{-9} and 10^{-6}M. Comparisons between baseline and post-treatment values that are significantly different are indicated with an asterisk (*).] (on right side of figure) indicates significant differences between pumping at different concentrations. (A) Mean data from 7 lymph vessels (B) Best fit line through mean pumping values at final 3 time points after each concentration of PMA (open circles, n=7) or 4-α-PMA (closed circles, n=7).*

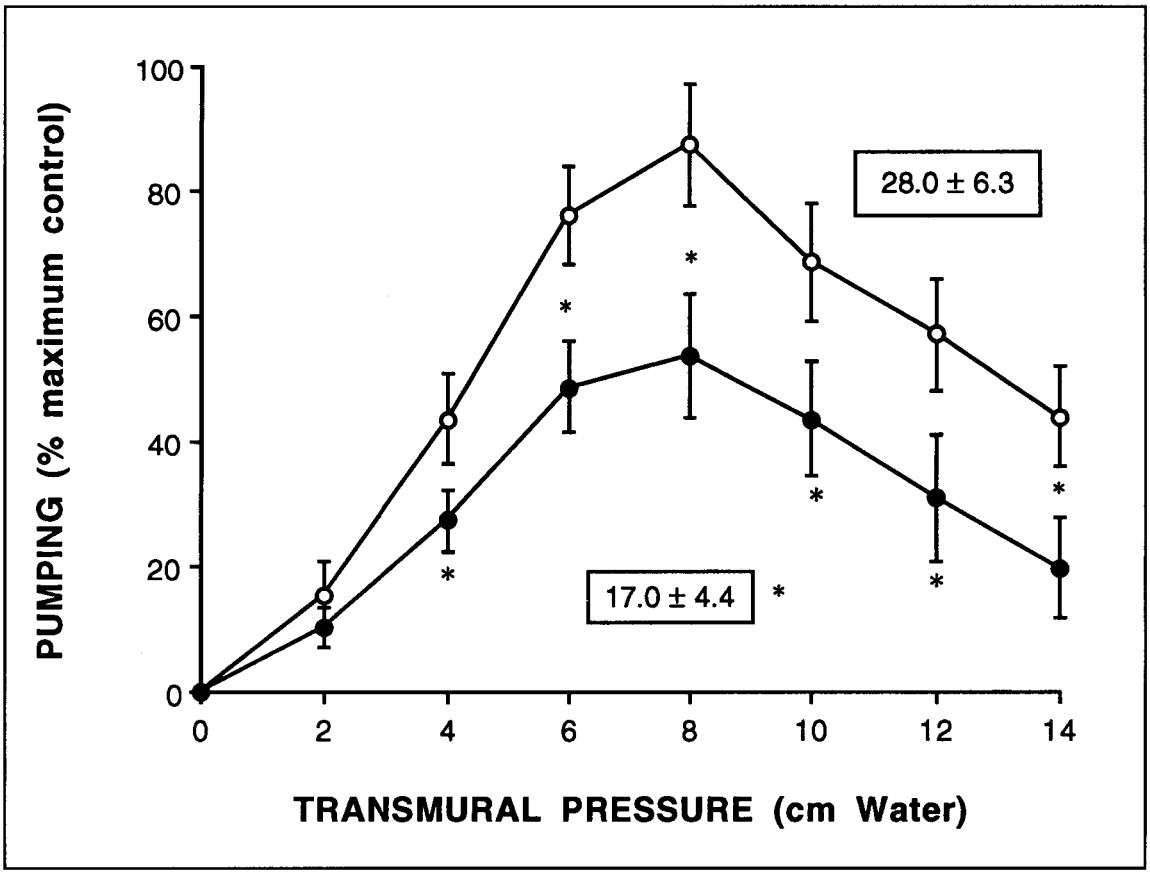

*Fig. 2. Effect of PMA ($5X10^{-8}M$) on the transmural pressure-pumping relationship (open circles before PMA added; closed circles, with PMA present, n=7). PMA depressed pumping significantly . * indicates $P < 0.05$ assessed with paired Students t-test. The numbers in the boxes represent the mean of the total volumes pumped over the range of transmural pressures tested before and after PMA was added to the vessels.*

collected at the outflow port every 10 minutes. The control hour was followed by an experimental period in which the factor to be tested was added to the Krebs solution in the inflow reservoir. In dose response experiments, each concentration of agent was administered for one hour prior to going on to the next higher concentration.

We also investigated the effects of several agents on the transmural pressure-pumping relationship using methods previously described (12). Two transmural pressure-pumping curves were generated for each vessel, one with Krebs and one with Krebs plus the agent to be tested. The transmural pressure was raised in 2 cm H_2O increments from 0 to 14 cm H_2O.

Chemicals

PMA (phorbol 12-myristate 13-acetate) and 4-α-PMA (4-α- phorbol 12-myristate 13-acetate) were purchased from Research Biochemical International (RBI - Natick, MA). Calphostin C and Chelerythrine were purchased from Calbiochem (La Jolla, CA). L-arginine and sodium nitroprusside (sNP) were purchased from Sigma (St. Louis, MO).

Analysis of Data

Differences in the diameter and in the pumping activity of the lymph vessels resulted in variations in the baseline fluid propulsion. To facilitate analysis, pumping was normalized in one of 2 ways depending on the protocol. In fixed pressure experiments, the initial six 10 minute values from the control hour were averaged and data for each 10 minute interval were expressed as a percent of this number. In variable transmural pressure experiments, all 10 minute interval flows were expressed as a percentage of the maximum pumping activity generated in the first control curve. All results were expressed as the mean ± SE. The results were analyzed with a single factor ANOVA or a group by time ANOVA with contrasts back to baseline. Where appropriate, the mean of the final 3 time points at each concentration of drug was compared with the mean of the final 3 values in the control period (i.e., before drug was added to the preparation), or with the values immediately preceding or following the period under investigation. In some cases, the data were assessed with a paired Students t-test. We interpreted $P<0.05$ as significant.

RESULTS

Effects of PMA on Lymphatic Pumping

Under fixed transmural pressure conditions, PMA, suppressed lymphatic pumping in a dose-related fashion (*Figs. 1A and B*). Using the raw data at 60, 120, 180, 240 and 300 minutes, PMA inhibited flow rates from a baseline average of approximately 8.7 ml/10 min, to 7.6, 5.1, 3.5 and 2.2 ml/10 min at 10^{-9}M, 10^{-8}M, 10^{-7}M and 10^{-6}M, respectively. The negative control (4-α-PMA) had no effect. The concentration of PMA that depressed pumping 50% was calculated to be 4.6×10^{-8} M (*Fig. 1B*). The effects of PMA were also investigated under conditions of changing transmural pressures. PMA (5×10^{-8} M) depressed pumping significantly when compared with the pressure-pumping curve obtained in the same vessel before the agent was added (*Fig. 2*). The total volume pumped over the duration of the experiment was calculated from the area under the raw data curves. Fluid output dropped from 28.0 ± 6.3 ml to 17.0 ± 4.4 ml when 5×10^{-8} M PMA was present, an inhibition of approximately 39%.

Effects of PKC Inhibitors on Lymphatic Pumping

Variable effects were observed when calphostin C was added to the lymph vessels at concentrations between 10^{-9} and 10^{-7}M. In some ducts pumping increased, in some there was little effect and in several others, flow declined. Overall, because of this variability, calphostin had no significant effect on fluid propulsion (*Fig. 3A*). Another PKC inhibitor chelerythrine, was tested between10^{-8}M and 10^{-6}M. This agent had a modest inhibitory effect on pumping which, however, was not significant (*Fig. 3A*). In addition, chelerythrine was tested in experiments in which pressures were varied between 2 and 14 cm H_2O but it had no significant effect on pumping under these conditions (*Fig. 3B*). The total volumes pumped over the duration of the experiment were 46.6 ± 8.9 ml before addition and 48.0 ± 10.4 ml after the addition of chelerythrine (10^{-6} M). However, the presence of 10^{-6} M chelerythrine attenuated significantly the inhibition of pumping induced with 5×10^{-8}M PMA (*Fig. 4*).

Effects of Sodium Nitroprusside or L-arginine on PMA-induced Inhibition of Pumping

The addition of PMA (5×10^{-8}M) to the vessels resulted in a prolonged and stable inhibition of pumping (*Fig. 5A*). At a concentration of 10^{-7}M, sNP completely reversed the PMA-induced depression in pumping (*Fig. 5B*). At a higher concentration of 10^{-6}M, sNP had little effect (data not

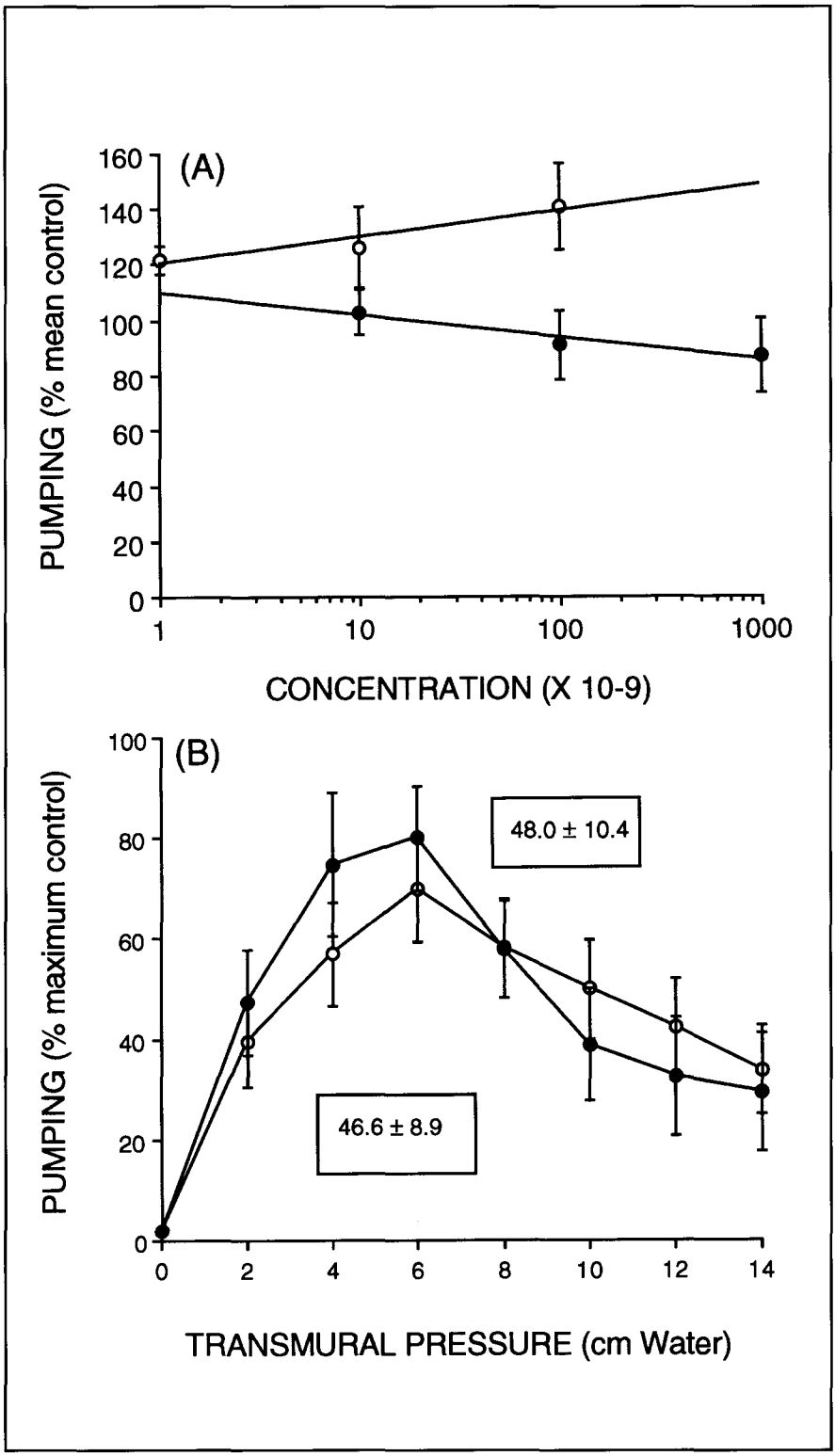

Fig. 3. Effects of PKC inhibitors on lymphatic pumping. Following a 1 hour control period, Calphostin C (open circles, n=11) or Chelerythrine (solid circles, n=11) was added to the reservoir. (A) The best fit line has been plotted through mean pumping values at final 3 time points after each concentration of inhibitor. Neither agent was found to have a significant effect on pumping. (B) Effects of Chelerythrine (10-6M, n=12) on the transmural pressure-pumping relationship (open circles before agent added; closed circles, with agent present). The numbers in the boxes represent the mean of the total volumes pumped over the range of transmural pressures tested before and after Chelerythrine was added to the vessels.

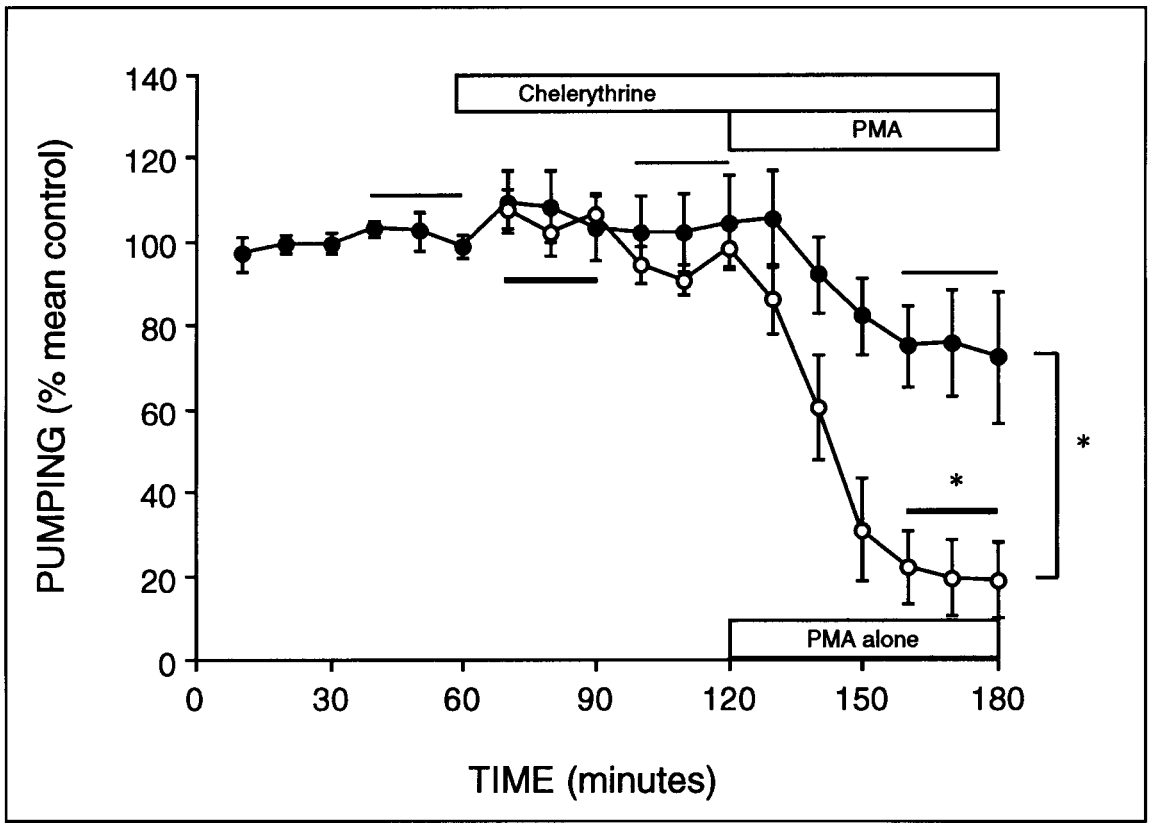

Fig. 4. Reversal of PMA's inhibitory effect with Chelerythrine. Effects of PMA alone are indicated with open circles (n=10). Closed circles denote experiments in which chelerythrine (10^{-6}M) was present between 60 and 180 minutes. PMA (5 X 10^{-8}M) was added at the 120 minute mark (n=10). Chelerythrine attenuated significantly the inhibitory effect of PMA. Comparisons between baseline and post-treatment values that are significantly different are indicated with an asterisk (*).] (on right side of figure) indicates significant differences between groups.*

illustrated). Similarly, L-arginine (10^{-4}M) was able to reverse partially the suppression in pumping observed with PMA (*Fig. 5C*).

DISCUSSION

As part of our continuing efforts to investigate the molecular and mechanical changes that regulate lymphatic fluid propulsion in response to lymph vessel stretch, we used pharmacological agents to investigate the role of PKC. The lymphatic myogenic response can be defined as the fluid propulsion induced by the application of a transmural pressure to the lymphatics. We assessed the effects of PKC stimulators or inhibitors under 2 experimental conditions. The first was the addition of incremental concentrations of test agent when the lymph vessel was stimulated to contract with a fixed transmural pressure. The second protocol involved a fixed concentration of test substance with the transmural pressure being raised incrementally. The latter result is perhaps a better indicator of the relationship between PKC activation and the myogenic response since it is the change in contractile activity in response to vessel stretch that has been accepted as the characteristic most commonly associated with myogenic activity (3).

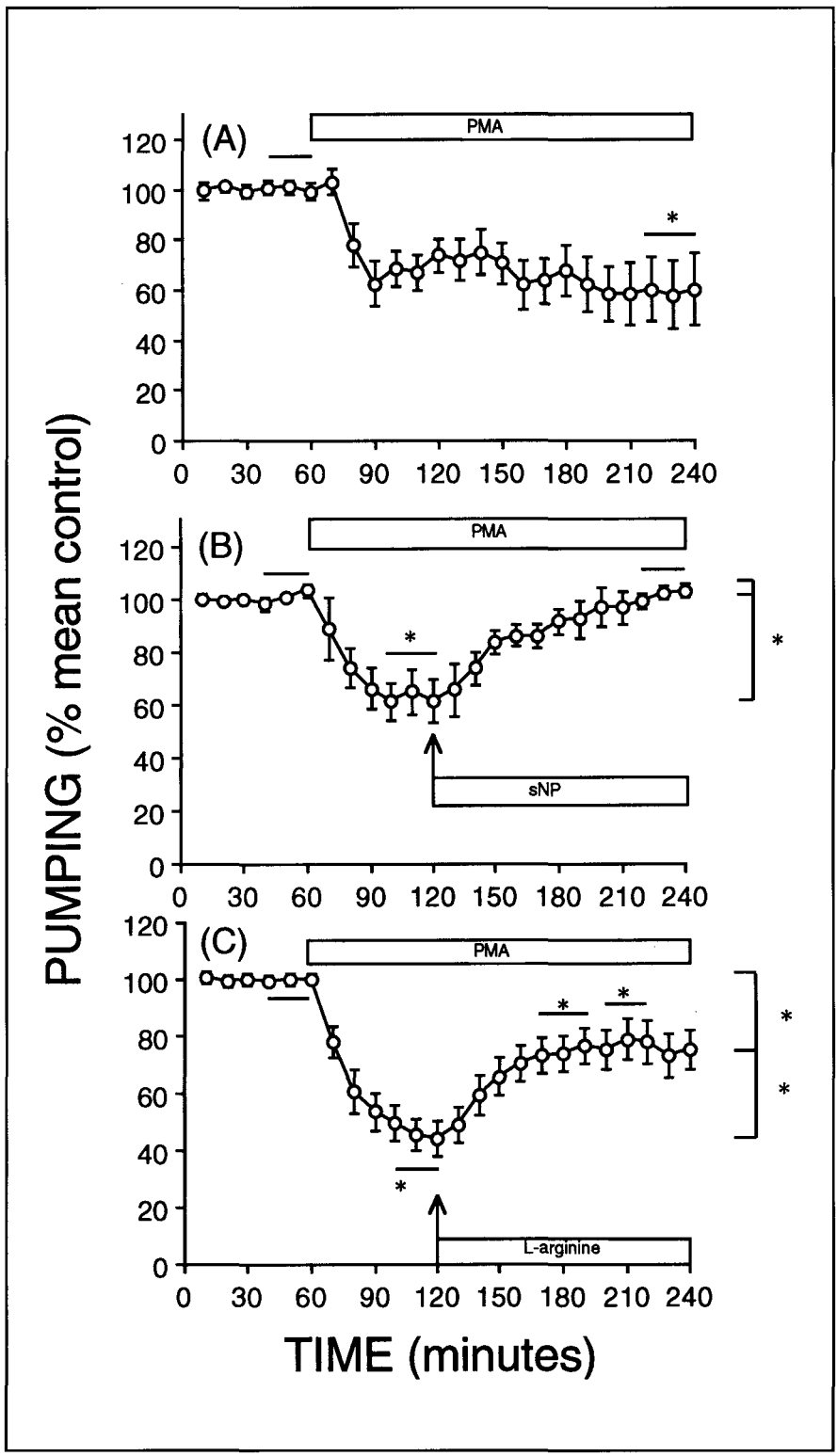

Fig. 5. Reversal of PMA-induced inhibition of pumping with sNP or L-arginine. (A) Effects of PMA alone (5×10^{-8}M, n=8) (B) Reversal of PMA's effect with 10^{-7}M sNP (n=7). (C) Reversal of PMA-induced inhibition of pumping with L-arginine (10^{-4}M, n=12). PMA inhibited pumping significantly in A, B and C. Following the addition of sNP, pumping returned to baseline (final 3 time points not significantly different from baseline). With the addition of L-arginine, a significant reversal of PMA's inhibitory effect was noted. Comparisons between baseline and post-treatment values that are significantly different are indicated with an asterisk (*).] (on right side of figure) indicates significant differences between pumping at different times.*

PMA, a known activator of PKC (13) had a marked effect on fluid propulsion in this preparation whereas 4-α-PMA, an inactive control compound, had no effect. Whereas the continuous presence of PMA in the lymph vessel preparation is unlikely to duplicate the transient temporal changes in PKC concentration that would be associated with the lymphatic contraction cycle, the data suggest that bovine mesenteric lymphatics contain PKC and that its activation has a negative effect on pumping.

One possible explanation for the inhibitory effect of PMA centers on the potential relationship between PKC and NO. Activation of PKC with phorbol esters inhibits purified NO synthase (14) and NO synthase in cultured bovine pulmonary artery endothelial cells (10). Inhibition of PKC increases the expression of ecNOS mRNA and the production of nitrogen oxides from bovine aortic endothelial cells (15). Therefore, in some blood and lymphatic vessels, PKC seems to have a negative influence on NO production. Previously, we demonstrated that inhibitors of NO including oxyhemoglobin, L-NMMA or methylene blue depressed lymphatic pumping activity and this suppression could be reversed with sNP or L-arginine (8). We concluded that NO facilitated the lymph vessel's response to stretch.

In the studies reported here, the inhibition of pumping induced by PMA could be reversed fully or partially with the administration of sNP or L-arginine. It is possible that PMA activated PKC which suppressed NO production within the lymphatic resulting in reduced pumping effectiveness. When NO was returned directly to the lymph vessels in the form of sNP, pumping was restored. Furthermore, when substrate was added, the lymph vessels were able to synthesize the appropriate amount of NO to elevate pumping activity. One might claim that the effects of sNP and L-arginine were independent of any relationship between PKC and NO. However, based on previous experience (8), sNP or L-arginine either inhibited or had no effect on lymphatic pumping respectively when tested alone. Only in NO-compromised lymph vessels, were increases in pumping observed with sNP or L-arginine.

There are of course, other potential ways that PKC could interfere with pumping activity. PKC modulates Ca^{2+} channel activity in blood vascular smooth muscle cells (16) and a similar effect may occur in lymphatics. Certainly, Ca^{2+} is important in the regulation of the lymphatic myogenic response (17). Arachidonic acid activates mitogen-activated protein kinases in vascular smooth muscle cells (18) and a relationship between PKC and arachidonate metabolites may also occur in lymphatics. Arachidonic acid and its metabolites have been implicated in modulating the lymphatic myogenic response (19). Clearly, additional studies need to be performed to elucidate the potential relationships between PKC, NO, Ca^{2+} and arachidonate metabolites in the regulation of lymphatic contractile activity.

To determine the effects of PKC suppression , we chose two inhibitors reputed to be specific for PKC, Calphostin C (IC_{50}, 50 nM) (20) and Chelerythrine (IC_{50}, 660 nM) (21). With calphostin C we observed that pumping increased in some lymphatics but overall, the results were variable and no significant effect could be determined. Similarly, with chelerythrine, we could not detect a significant effect on fluid propulsion although with this agent, there was a tendency for flow to decline.

Since PKC activation depressed lymphatic pumping, we expected that inhibition of PKC would enhance fluid propulsion. There are several ways to interpret the lack of effect of PKC inhibitors on lymphatic pumping. The most obvious conclusion is that PKC is not involved in regulating the myogenic response to lymph vessel stretch. PKC activation in lymphatics may have more relevance to agonist-induced changes in contractile activity. For example, in blood vessels, endothelin (22) and norepinephrine (23) exert at least part of their effects through

the activation of PKC and both of these compounds are known to affect lymphatic contractile activity (24, 25). Therefore PKC may be important in modulating the lymphatic response to humoral or neurogenic factors.

An alternative hypothesis for the lack of effect of the PKC inhibitors can be built around a potential relationship between PKC and NO. Previous studies from our laboratory suggest that NO is one of the important transduction factors that regulate contractile activity (8). Data from the literature suggest that NO exerts a negative feedback control of PKC activation (26). If this finding is applicable to lymphatics, one might postulate that NO formed as the vessel is stretched facilitates pumping activity. As part of this response, NO exerts a negative effect on PKC restricting the amount available in the smooth muscle. This explanation could account for the fact that inhibitors of PKC had little impact on pumping. However, when certain agonists come in contact with the lymphatic vessel, PKC is upregulated and this in turn, suppresses NO production and inhibits pumping. In future studies, measurements of PKC and NO in the lymphatic vessel may help to elucidate the potential interaction between these factors.

In summary, our data demonstrate that activation of PKC in bovine mesenteric lymphatic vessels results in a suppression of pumping activity that may be mediated in part, by an inhibitory effect on NO. The inability to demonstrate a significant effect of PKC inhibitors in this preparation suggests that PKC is not involved in regulating lymphatic response to changes in transmural pressure.

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