Pflügers Arch – Eur J Physiol (1996) 431 : 618–624

ORIGINAL ARTICLE

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Atrial natriuretic peptide attenuates flow in an isolated lymph duct preparation

Received: 19 May 1995/Received after revision: 4 October 1995/Accepted 9 October 1995

Abstract We investigated the hypothesis that atrial natriuretic peptide (ANP) attenuates lymph vessel pumping. In the present experiments, isolated bovine lymphatic vessels were cannulated at each end to create inflow and outflow ports for the administration of Krebs' solution (vehicle) or ANP and for the measurement of fluid pumped by the vessel respectively. Once cannulated, the vessels were placed in a temperature-regulated bath circulated with oxygenated vehicle. Transmural pressure was regulated by the height of a fluid-filled reservoir. Lymph pump activity was assessed by measuring the volume of outflow every ten minutes. ANP was administered at concentrations of 0 (control), 0.1, 1.0, 10 and 100 nM. Data were expressed as a percentage of the value in the control period. When compared with vehicle, ANP produced a significant inhibition of lymph pump activity by 7.7% at 0.1 nM, 24.2% at 10 nM and 38.26% at 100 nM averaged over the hour for which the vessels were exposed to each concentration. Thus ANP inhibits lymph pumping concentration dependently. This may be yet another mechanism by which ANP exerts its haemodynamic effects.

Key words ANP · Lymph pump · Bovine lymphatic ducts

Introduction

It has been postulated that atrial natriuretic peptide (ANP) causes a shift of fluid out of the capillary into the interstitium [4, 26]. This is based upon the observation that ANP stimulates simultaneously a reduction in blood pressure and an increase in haematocrit and plasma protein concentration [16], even in the absence of renal fluid loss [1, 25]. The mechanism for this effect is not yet established, but one hypothesis is that ANP alters capillary permeability [19] possibly brought on by an increase in endothelial cell permeability [28]. However, others have concluded that ANP has no effect on microvascular permeability [11, 24]. Indeed such discrepant observations may be due to the vascular compartment being studied. For example, Peters et al. [22] found a redistribution of blood volume with an increase to the intestine but a reduction in liver and heart and concluded that the effect of ANP may differ among vascular beds.

An alternate hypothesis for the non-renal loss of fluid in response to the administration of ANP is a change in microvascular hydrostatic pressure [6, 9]. Yet again, the evidence is inconclusive. Since the same species was used in both these studies perhaps the disparate results are due to either a difference in the techniques used to measure vascular resistance (direct visualization [6] v. microsphere injection [9]) or specific to the vascular bed being studied. Thus, how ANP promotes the transudation of fluid to the interstitium is still controversial. We have focused instead on a putative biologic effect of ANP that has received limited investigation.

In light of the significance of the lymphatic system in restoring extravasated fluid back to the intravascular compartment [5] we investigated the effect of ANP on lymphatic pumping. While a few attempts to study this have been made, the results are inconclusive. Ohhashi and coworkers [21] measured tension in isolated lymph vessel rings and found that ANP relaxed vessel rings precontracted with bradykinin. Furthermore, they were also able to demonstrate that ANP produced a dose-dependent reduction in the rate and the amplitude of lymph vessel contractions.

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Unfortunately since the main function of the lymphatic system is to actively pump fluid collected in the interstitium and restore it to the intravascular compartment, a change in lymph vessel tone or in the activity of a ring preparation is not necessarily a reliable indicator of lymph pump function. It is the measurement of lymph flow that is the more physiologic indicator of lymphatic pumping activity. Another group [11] studied the effect of ANP on lymph flow. Using an elegant dog forelimb preparation, they found that ANP had no effect on lymph flow or protein concentration. However, their experiments were performed under the condition of constant vascular perfusion pressure and by their own admission they could not predict similar results under the more physiologic conditions of pulsatile vascular perfusion pressure.

We speculated that while ANP may act at the level of the capillary it's effects may extend to include attenuating lymph pump activity thereby causing sequestration of fluid in the interstitium. We tested our hypothesis that ANP inhibits the lymph pump in isolated bovine lymph vessels. Our preparation allowed us to measure the volume pumped by actively contracting lymph vessels. Our results show that at a constant transmural pressure, ANP attenuates lymphatic pumping concentration dependently.

Materials and Methods

Preparation

As previously described [12, 18] bovine lymphatic vessels (> 2 mm diameter) were isolated from the mesentery within 10 min of slaughter. Vessels 8–10 cm in length, consisting of three or four lymphangions, 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 0.01% Evan's blue dye (in lactated Ringer's injection U.S.P., Baxter) to a transmural pressure of 10–12 cm H_2O. Leaky vessels were discarded. No more than two vessels were used from a single mesentery.

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). The outer chamber of the organ bath was filled with distilled water that was circulated through a heated water bath (Tempette TE-8D, Mandel Scientific). This provided the heat source for maintaining the temperature of the solutions bathing the vessels, both intra-and extraluminally. Temperature and pH of the circulated fluid were maintained at $37.8 \pm 0.0°$ C and at 7.42 ± 0.02 (ϕ12 pH/ISE meter, Beckman Instruments) respectively.

The inflow ports for each lymph vessel were arranged in series and were connected to a common reservoir (10 ml volume) that contained either vehicle (Krebs' solution) which served as the time control series of experiments or ANP. The vessels were arranged in series with respect to the common reservoir and in parallel with respect to each other. The fluid level in the common reservoir was kept constant by means of inflow and outflow ports connected to a roller pump (Masterflex, model 7567–70, Cole-Parmer). This fluid was replenished from a larger supply at a rate of 10 ml/min and supplied a maximum of six vessels simultaneously. 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 centimetres) determined transmural pressure. By adjusting the height of the fluid in the 10 ml reservoir and the outflow port from each vessel simultaneously in relation to a zero reference point (fluid surface of the bath) transmural pressure was manipulated.

An hydrostatic pressure of 2–3 cm H_2O was applied for 10–20 min upon placement of the vessels into the bath chamber. This gently distended the vessels and perfused them with fresh solution. After this period, the hydrostatic pressure was removed and transmural pressure was set at 6 cm H_2O, whereupon the vessels resumed pumping and were allowed a further 1 h to equilibrate. Vessels that did not resume pumping within this hour were discarded. A transmural pressure of 6 cm H_2O was maintained throughout the dose/response experiments. Earlier work in this laboratory has demonstrated that a transmural pressure of 6 cm H_2O elicits submaximal flow [12]. This permitted us to observe changes in pumping in either a positive or negative direction.

Protocol

The experimental protocol consisted of a 1-h control period during which vehicle was presented to the vessels. The volume pumped was calculated from the weight of the fluid collected at the outflow port every 10 min over 1 h. The six to 10-min values were averaged and subsequent data were expressed as a percentage of this number. Since vessels from different animals may vary in size, normalizing the data in this manner corrected for differences in flow. For example, the average volume pumped during the control hour ranged from 4.07–28.42 ml/10 min in the vehicle group to 2.87–20.39 ml/10 min in the ANP group. The control hour was followed by an experimental period. Krebs' solution was replaced by ANP (human ANP, A-1663, Sigma [27]) dissolved in Krebs' solution at increasing concentrations (0.1, 1.0, 10 and 100 nM). Each concentration was administered for 1 h prior to proceeding the next higher concentration. A separate group of experiments receiving only vehicle served as a time control.

The flow through a lymphatic vessel can be influenced by the pressure difference across the vessel wall. The relationship between flow and pressure can be described by a bell-shaped curve and can be altered by various agents [13]. We also studied the effect of ANP on the optimal vessel wall distending (transmural) pressure at which the lymph vessel pumped. To do this, we lowered or raised both the inflow (common 10 ml reservoir) and the outflow ports simultaneously in relation to the fluid level in the organ bath. In this series of experiments vessels were prepared as above and allowed a 1-h equilibration period to resume pumping at a transmural pressure of 6 cm H_2O. After this hour the transmural pressure was lowered to zero in 2-cm decrements at 5-min intervals. After 5 min of equilibration at the new pressure, the fluid pumped was collected for 10 minutes before increasing the transmural pressure by 2 cm H_2O and repeating the equilibration/collection time periods. In this manner, the effect of 100 nM ANP was tested at transmural pressures of 0–14 cm. Flow for the ANP and vehicle groups was calculated as a percentage of the maximum flow observed during the administration of vehicle. Two transmural pressure curves were obtained for each vessel: one a control curve in the presence of vehicle, the other in the presence of ANP. The order in which the curves were performed was randomized.

Statistical analysis

Results obtained with each concentration of ANP were compared with the vehicle time control by a one-way analysis of variance as well as with the pre-ANP control hour by a repeated-measures analysis of variance followed by a post-hoc test (Bonferroni) when the *F*-statistic of the analysis of variance was significant (SigmaStat,

Jandel Scientific). The data were grouped and analysed at intervals of 1 h to coincide with the concentrations of ANP that were administered. Significant differences refer to the mean values obtained during the hour.

The transmural pressure curves were analysed by calculating first the area under the control and experimental curves and then performing a paired *t*-test. The order in which transmural pressure curves were obtained, ie. control first or experimental first, was analysed for differences by an unpaired *t*-test prior to combining the data. A significant difference was interpreted when $P < 0.05$. Data plotted are mean ± SEM.

Results

The viability of our preparation was confirmed by the return of spontaneous contractions to the cannulated lymph vessels. Of a total 62 vessels cannulated, 43 vessels resumed pumping by the end of the equilibration period. Figure 1 illustrates the stability of the preparation. Vessels in the vehicle group maintained flow at a steady rate for the 5-h duration of our experiments, though the variability in lymph pump activity increased gradually with time (180–300 min; Fig. 1). This was due in part to a reduction in pumping by some vessels (2 out of 14 vessels reduced pumping by 85–90%), and in part to an increase in pumping by others (2 out of 14 vessels increased pumping by 60–65%). Nevertheless, when the data were grouped by 1-h intervals and analysed by a repeated-measures analysis of variance no significant differences were found.

Data collected prior to the administration of ANP (0–60 min) served as an internal control. These values were not significantly different from 0–60 min in the vehicle group. A cumulative dose/response curve (Fig. 1) showed that ANP ($n = 12$) attenuated the volume of fluid pumped by the lymphatic vessels at 0.1, 10 and 100 nM when compared with a second group of vessels that served as an appropriate vehicle time control ($n = 14$). ANP also influenced significantly lymph pumping activity at 10 and 100 nM when compared with the hour prior to the administration of ANP. While the analyses demonstrated significant differences over all, post-hoc tests at individual time points were not significant, indicating an effect overall, but no effect at any individual time. ANP at 0.1 nM did not influence significantly lymph pumping when compared with the pre-ANP hour. ANP at 1.0 nM did not alter pumping activity when compared with either the vehicle time control or the hour prior to the administration of ANP.

An effect was seen within the first 10 min of presenting ANP to the vessels with maximum inhibition occurring by 20 min. Figure 2 illustrates the maximum inhibition in pumping achieved at each concentration of ANP when compared with vehicle. After 20 min, ANP's inhibitory effect was attenuated with some data approaching a return to pre-ANP levels. During the administration of 0.1 nM ANP, the maximum inhibition in pumping occurred at 30 min.

The average response to ANP was a reduction in lymph pump activity. The biological variability existing between vessels from different animals is shown in Fig. 3. These two vessels represent the range of responses seen in the presence of ANP. They were selected for illustration for two reasons: firstly, the experiment on

Fig. 1 Fluid pumped by the lymph vessels expressed as a percentage of the flow in the 1st hour control period. Each *line* represents the administration of atrial natriuretic peptide (ANP) at increasing concentrations (0.1, 1.0, 10 and 100 nM respectively) to the experimental group (*closed circles*, $n = 12$). The vehicle group (*open circles*, $n = 14$) served as a time control $^{*}P < 0.05$ ANP group compared with the vehicle group by ANOVA $^{\#}P < 0.05$ for comparison of 0.1, 1.0, 10 and 100 nM ANP were compared with 0 nM ANP

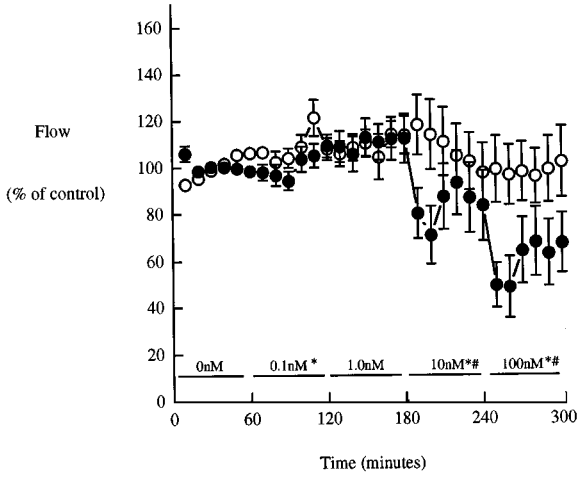
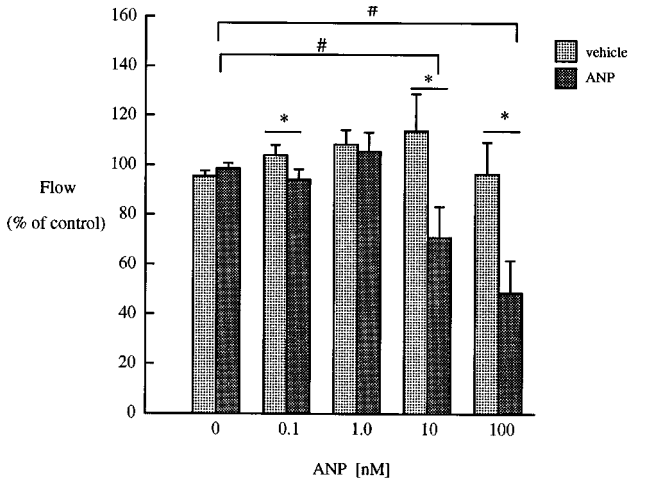


Fig. 2 The maximum inhibition in pumping occurred within 20 min of exposure to ANP. The exception was 0.1 nM where pumping was maximally inhibited by 30 min. Flow is calculated as a percentage of the average volume pumped during the 1st hour (pre-ANP). The pre-drug values (0) for the ANP and vehicle groups were not significantly different. *Lighter bars* = vehicle, *Darker bars* = ANP. Statistical differences based on analysis of data grouped at 1-h intervals $^{*}P < 0.05$ ANP group compared with the vehicle group by ANOVA $^{\#}P < 0.05$ for comparison of 0.1, 1.0, 10 and 100 nM ANP with 0 nM ANP



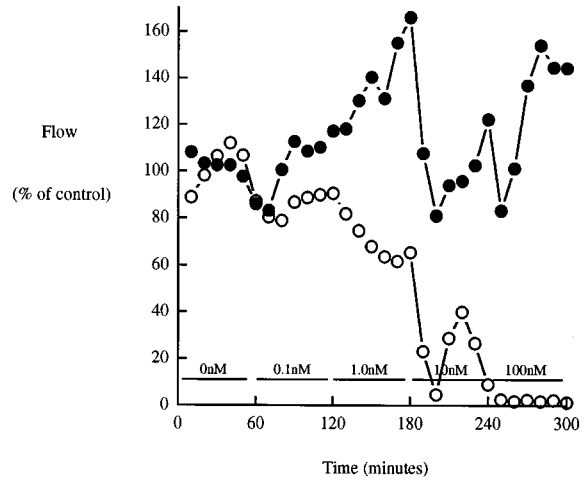


Fig. 3 Examples of two individual lymph vessel responses to ANP administration illustrating the biological variability that can be seen between animals. Such variability has been seen with other agents such at nitric oxide [10]. Both vessels have a similarly stable control hour (0 nM), however upon exposure to ANP their responses diverge

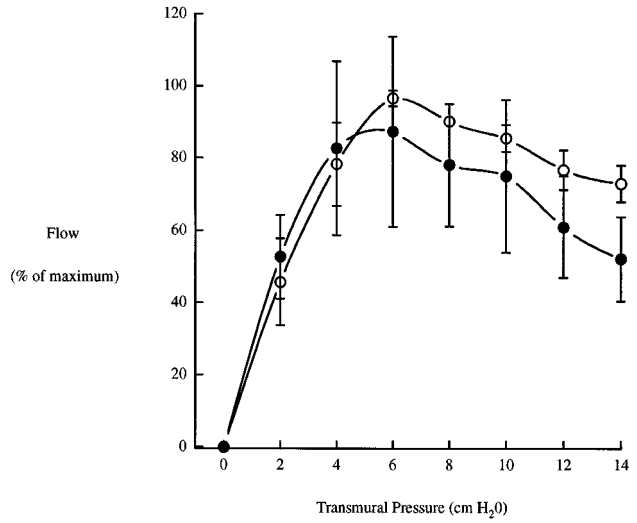


Fig. 4 Fluid pumped by the lymph vessels in response to a series of changes in transmural pressure. Values (mean ± SEM) are expressed as a percentage of the maximum volume of fluid pumped during vehicle administration (*open circles*, $n = 8$). The administration of 100 nM ANP (*closed circles*, $n = 8$) did not alter pumping activity. The areas under each curve, as determined by a paired t-test, were not significantly different

each vessel was performed on the same day and, therefore, under identical conditions and secondly, the data obtained during the 1st hour control period were similarly stable in both vessels. Yet when exposed to ANP the flow from one vessel (open circles) was attenuated completely by 100 nM ANP while the flow from the second vessel (closed circles) was not attenuated and at some concentrations was increased. Of the 12 vessels tested in the experimental group, 66% (8 out of 12) responded with a reduction in pumping and flow while 33% (4 out of 12) responded with either no change or an increase in pumping and flow when exposed to sequentially increasing concentrations of ANP.

Transmural pressure curves were obtained during the administration of 100 nM ANP ($n = 8$, Fig. 4) and were compared with curves obtained in the presence of vehicle. The order in which vehicle and ANP were presented to the vessel did not influence the pumping response to a change in transmural pressure (vehicle first vs. vehicle last $P = 0.22$; ANP first vs. ANP last $P = 0.69$). Therefore when studying the pressure/flow relationship the data were combined and further analysis did not distinguish between vehicle or ANP being presented first to the vessels. Exposure of the vessels to 100 nM ANP did not alter the volume of fluid pumped (58.98 ± 9.64 ml for the vehicle group compared with 49.88 ± 10.69 ml for the ANP group, $P = 0.35$). In addition, ANP did not alter the overall shape of the pressure flow curve nor did it influence significantly the transmural pressure at which maximum flow occurred when compared with the curves obtained during the administration of vehicle.

Discussion

As a result of spontaneous contractile activity, lymph vessels are capable of returning capillary transudate to the intravascular compartment. The intrinsic pumping capability of lymphatic vessels suggests a role in the maintenance of intravascular volume and haemoconcentration. Furthermore, the changes in blood pressure and haematocrit associated with the administration of ANP [16] imply that ANP may interfere with the effectiveness of the lymph pump leading to fluid being sequestered in the interstitium rather than being returned readily to the intravascular space. Yet studies of interactions between ANP and the lymphatic system are limited and inconclusive. The isolated lymph duct preparation described herein allows us to study the interaction between ANP and the lymph pump.

Our present results agree with Anderson et al. [3] who measured the frequency and force of contractions in small (2–3 cm) segments of immature ovine mesenteric lymphatic vessel, but who did not measure flow through the vessel. They found that ANP, at concentrations similar to those used here, attenuated both the frequency and force of contractions concentration dependently. We have extended their earlier conclusions by demonstrating that an ANP-induced reduction in contractility of a lymph duct can be translated into a physiologically significant event such as a reduction in flow through the vessel.

In our dose/response experiments (Fig. 1) vessels were exposed sequentially to increasing concentrations of ANP. An effect was elicited within the first 10 min of ANP being presented to the vessel. Maximum inhibition in flow usually occurred by 20 min (Fig. 2), after

which the magnitude of ANP's effect declined. The apparent tachyphylaxis observed after 20 min exposure to ANP may be due to down regulation of ANP receptors, a speculation supported by Frank et al. who have shown that ANP receptors on bovine brain capillary endothelial cells in culture are down regulated after 20 min exposure to ANP [14].

This temporal pattern was repeated at each concentration of ANP including 1.0 nM, but not at 0.1 nM ANP. At the lowest concentration of ANP maximum inhibition occurred at 30 min (Fig. 2). While even this low concentration (0.1 nM) of ANP produced a statistically significant difference compared with its time control (60–120 min) this effect may have been due to an increase in pumping within the vehicle group. Yet the significance of an increase in pumping in the vehicle group is uncertain since there was no statistical difference when compared with its internal control (0–60 min, Fig. 1).

The route by which ANP may reach the lymphatic system is uncertain. The movement of ANP from the microcirculation to the lymphatic system has yet to be investigated. The molecular weight of the circulating form of ANP is less than 3,000. It is therefore possible that ANP may leave the intravascular space at the level of the capillary, diffuse into the interstitium where it would be removed and transported back to the intravascular space via the lymphatic system. Alternatively ANP may exert its effect on the lymphatic system by that system's own blood supply, the vasa lymphorum, which supplies the media and adventitia of larger lymph ducts such as those used in our study [20]. Acting via this latter route ANP may influence lymphatic smooth muscle.

While our preparation allowed the administration of agents both intra- and extraluminally, in this series of experiments we focussed on the effects of ANP via the intraluminal route. Our rationale considered that if ANP entered the interstitium to exert an effect extraluminally this would influence primarily initial lymphatics. The vessels used in the present study were located higher in the lymphatic tree (mesentery) and as such would not be exposed to significant concentrations of ANP extraluminally.

The lack of an effect during the administration of 1.0 nM ANP is surprising, especially in conjunction with the statistically significant response during the administration of 0.1 nM ANP and the pronounced initial response to 10 nM ANP when compared with vehicle. Indeed, since the half-maximal activation of guanylate cyclase by ANP occurs at 1–10 nM [2] one would have predicted 1.0 nM ANP to produce a biologic effect. Nevertheless, the trend in lymph pump activity throughout the duration of 1.0 nM ANP is similar to that obtained with the other concentrations.

The concentrations of peptide used in our study represent a range commonly seen in the literature. A concentration of 0.1 nM is equivalent to plasma levels of ANP in humans in response to a 20% volume expansion [17]. The higher concentrations of ANP used here are more representative of pharmacologic doses, or of concentrations that may be seen in pathological states such as congestive heart failure [7], cirrhosis with ascites [8] or hypertension [15]. Furthermore, this range of concentrations encompasses those concentrations necessary to produce half-maximal activation of guanylate cyclase ex vivo [2].

In addition, any agent that influences lymphatic pumping when transmural pressure is held fixed, as in our dose response experiments (Fig. 1), may also influence the shape of the curve that describes the relationship between transmural pressure and flow. Such changes include an increase or decrease in the overall height of the curve, as well as a shift in the optimal operating pressure. For example, haemoglobin attenuates dramatically lymphatic pumping and flow through the vessel. It also stimulates a rightwards shift in the pressure/flow curve so that maximum flow is not only attenuated, but occurs at a higher pressure compared with vehicle [13]. Therefore, we investigated the possibility that ANP might also influence the pressure/flow curve (Fig. 4).

When transmural pressure was manipulated in the presence of 100 nM ANP there was a slight reduction in flow as pressure increased, however, the data obtained at a transmural pressure of 6 cm H_2O (Fig. 4) were not of the same magnitude of lymph pump inhibition as that obtained under similar conditions in the dose response experiments (Fig. 1). The explanation for this discrepancy is twofold: it involves the methodology used for each series of experiments and the regulation of ANP receptors. The dose response experimental protocol consisted of the administration of increasing concentrations of ANP at 1-h intervals. With this protocol we observed that ANP had an effect which was attenuated with continued exposure to the peptide, but that the attenuation was overcome by increasing the concentration of peptide. On the other hand, the method for conducting the pressure flow experiments required that the peptide be administered for 15–30 min prior to the collection of data. In addition, the equilibration and data collection times required a total of 15 min at each transmural pressure. Thus vessels were exposed to ANP for 45 min before a transmural pressure of 6 cm H_2O was reached and the results recorded. If, as shown by Frank and colleagues [14], ANP receptors may be downregulated within 20 min exposure to ANP and since, in this study, ANP's maximum effect occurred within the first 20 min of exposure to ANP and was thereafter attenuated, it is possible that the vessel receptors were desensitized to ANP or down regulated by the time data collection for the transmural pressure experiments began. Temporal factors may thus have prevented us from seeing any effect of ANP on the pressure flow relationship.

Alternatively, the ineffectiveness of ANP in this setting may be due to the intrinsic contractile nature of lymph vessels, which function according to the Starling principle i.e., the force of contraction depends upon preload and afterload [23]. In an in vitro setting such as ours, preload of the lymph vessel is manipulated by changing transmural pressure. If activity of the lymph pump is influenced by a mechanical stimulus such as wall stretch it is possible that the biochemical stimulus provided by ANP is attenuated when a change in transmural pressure elicits a potent contractile response from the vessel wall that supercedes the inhibitory biochemical effects of ANP. Since flow may be altered transiently immediately after a change in transmural pressure, we adjusted for this by allowing a 5-min equilibration period prior to the collection of data. This transient change, however, may have provided the stimulus for an increase in the force of contraction which could not be controlled. Since 100 nM ANP continued to depress pumping by 30–35% during the last half of its administration in the presence of a constant transmural pressure (Fig. 1), yet had no similar effect when transmural pressure was manipulated, this may be the most likely explanation for the discrepancy in our data.

In conclusion, despite the observation that ANP had a limited effect on the pressure flow relationship in our preparation, ANP did have an effect on lymph pump activity; it attenuated lymphatic pumping concentration dependently . Therefore an interaction with lymph pump activity may contribute to the haemodynamic effects of ANP by sequestering fluid in the interstium, rather than promoting its return to the intravascular space. It is worth noting that the observed tachyphylaxis suggests that this effect is time dependent and that the mechanisms that regulate ANP receptors may play a significant role.

Acknowledgements The authors wish to thank Mel Walker and Ryding-Regency and Piave Meat Packers for their co-operation. Funding for this project was provided by a Medical Research Council of Canada grant to MGJ. DJA is supported by a personnel development fellowship from the Ontario Ministry of Health.

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