Calcium Dependence of Flow-Induced Dilation
Cooperative Interaction With Sodium

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Acetylcholine causes dilation of small arteries by releasing endothelium-derived relaxing factor or factors (nitric oxide [NO] or a nitric oxide-like substance) and possibly other molecules from the endothelium.1-3 Intraluminal flow can also cause small artery dilation, which has been reported to be entirely4-6 or partly7 endothelium dependent. In the latter case, part of the flow-induced response originates from subendothelial sites in the extracellular matrix, the smooth muscle cells, or both. Other differences and also similarities between flow- and acetylcholine-induced dilation have been reported. Flow, but not acetylcholine, dilation is sensitive to reduction in extracellular Na+ concentration.8 Both are reduced by hemoglobin and methylene blue and are potentiated by superoxide dismutase and cyclic guanosine monophosphate phosphodiesterase inhibitors.9,10 Acetylcholine and the endothelium-dependent component of flow dilation are prevented by substances that influence the synthesis of NO from arginine9,11; the endothelium-independent component is not.12 Both acetylcholine and flow relaxation are reduced by amiloride.13,14 When observed in isolated vessels from several beds, neither appears to be influenced by indomethacin.7,10

The endothelial dilation caused by acetylcholine in most cases is dependent on the presence of external Ca2+.15,16 (for an exception, however, see Reference 17). This may reflect the Ca2+ dependence of NO production, which is optimal at 1.2 mM.18 The sensitivity of the dilation to flow has not been explored in detail.

Experiments were carried out to compare the sensitivity of flow-induced and acetylcholine-induced dilation in a resistance branch of the rabbit central ear artery to changes in extracellular Ca2+. Furthermore, because Na+ and Ca2+ interact in a number of processes in the artery wall related to vascular tone and because flow but not acetylcholine-induced dilation is sensitive to a reduction in Na+ (145–120 mM), the consequences of lowering both Na+ and Ca2+ on these dilator responses were investigated.

The lowering of Ca2+ reduced both modes of dilation—that induced by flow and by acetylcholine; raising Ca2+ depressed flow dilation conspicuously more than acetylcholine dilation. The reduction in flow dilation resulting from lowering the sodium concentration was offset by a concomitant or subsequent reduction in Na+. By comparison, the reduction in acetylcholine dilation caused by a lowering of Ca2+ was not influenced by a concomitant reduction in Na+.

These findings emphasize that the flow-induced dilation involves processes different from that induced by acetylcholine and suggest that the role of these ions in flow-related modulation of tone may depend not only on their absolute but on their relative concentrations.
Methods

Branches of the central ear artery of the rabbit (approximately 200 μm o.d.) were mounted in a resistance artery myograph19,20 at a length optimum for agonist contraction and were maintained at 37°C in physiological saline solution (PSS) of the following composition (mmol/l): Na+ 144.9, K+ 5.9, Ca2+ 1.6, Mg2+ 1.2, Cl− 134.7, HCO3− 14.9, SO4− 1.17, dextrose 22.2, and EDTA 0.026 equilibrated with 95% O2−5% CO2. Only two artery segments were dissected from the same rabbit, and these were usually obtained adjacent to each other on the same branch. Bath PSS could be infused into the lumen of the mounted artery with a syringe infusion pump (model 22, Harvard Apparatus, South Natick, Mass.) through a glass micropipette whose tip was placed just within the open end of the segment. The end of the micropipette occupied 60−80% of the area of one of the open ends of the stretched segment. After an equilibrium period of 1 hour, wall tone was increased by adding to the tissue bath norepinephrine (10−6 M), which resulted in an increase in tone of approximately 60−70% of tissue maximum, and then the relaxation to cumulative additions of acetylcholine (10−4, 10−5, 10−6 M) was recorded. These were carried out to assess the vascular smooth muscle and endothelial integrity of the preparation. Then the tissue was washed several times, and after an equilibrium increase in tone to norepinephrine (10−6 M) was reached, relaxation to the cumulative additions of papaverine was recorded. After the preparation was washed several times over a period of at least 30 minutes, wall tone was again increased with norepinephrine (10−6 M), and the relaxation to 3−5 minutes of intraluminal infusion of PSS (20 μl/min) also containing norepinephrine (10−6 M) was initiated. In previous experiments, this was found to cause an approximately half-maximal flow dilation. This infusion usually caused a rapid loss of wall tone, which was maintained as long as the infusion was continued and which reversed on its cessation. After consistent responses to intraluminal flow were obtained, a length optimum for agonist contraction and were maintained at 37°C in PSS were made, and 30 minutes later both dilator responses were repeated.

Increases and decreases in Ca2+ were achieved by changing the concentration of CaCl2 without altering other constituents of the PSS. Decreases in Na+ were accomplished by reducing the NaCl in the PSS with equivalent substitution by N-methyl-D-glucamine, after which the PSS was brought to pH 7.4 with hydrochloric acid. Na+ was reduced from the normal level of 145 mM to 132 and 119 mM. This represents a 10% and 20% diminution of the added NaCl to the PSS. This solution is iso-osmotic with the control PSS.

Responses to norepinephrine are expressed as percent increase in wall force and dilation as a percent change in preflow level of wall tone; both are represented as percentage change from control measurements.

Statistics

All results are reported as mean±SEM. Tests of significance were made using the Student's t test for grouped single and paired data as appropriate. A value of p<0.05 was considered statistically significant.

Results

Effect of Increases and Decreases in Ca2+ on Flow, Acetylcholine, and Papaverine Relaxation

The Ca2+ concentration in the PSS was reduced to 90% and 80% of normal level, to 1.44 and 1.28 mM, respectively, and increased to 120%, 140%, 200%, and 400% of normal, to 1.92, 2.24, 3.20, and 6.40 mM, respectively (Figures 1 and 2). Flow relaxation of the resistance branch of the rabbit central ear artery was reduced by both the increase and decrease in Ca2+ concentration. At 80% of normal Ca2+ levels, flow relaxation was 27.7±5.4% (n=7), and at 140%, 50.4±12.2% (n=6) of control. Comparable changes occurred in the relaxation to acetylcholine (3×10−8, 10−7 M) when calcium was reduced. At 80% of normal Ca2+ levels, the relaxation induced by these two concentrations of acetylcholine was 47.1±12.1% (n=6) and 3.7±14.2% (n=6) of control, respectively. Increases in calcium did not change the acetylcholine dilation. At 200% and 400%, relaxation was 105±12% (n=4) and 98.0±7% (n=4) of control, respectively.

There was no significant change in the contraction to norepinephrine (10−6 M) and the dilation to papaverine (1 and 3×10−7 M).

Simultaneous Proportionate Reduction in Ca2+ and Na+ on Flow, Acetylcholine, and Papaverine Relaxation

Reductions in either Na+ or Ca2+ alone significantly attenuate flow dilation8 (see above). However, when Ca2+ and Na+ represented by NaCl in the PSS were both reduced proportionately up to 70% of control, N-methyl-D-glucamine being used as a substitute for Na+, the size of the flow-induced dilator response of the resistance artery did not significantly change (Figure 3). The reduction in the muscarinic response resulting from lowering of Ca2+ (see above) was not influenced by the concurrent proportionate reduction in Na+ (Figure 4).
FIGURE 1. Line graphs show effect of decrease and increase in calcium concentration in physiological saline solution (PSS) on dilation of segments of resistance branch of rabbit ear artery due to flow (20 μl/min) and acetylcholine (Ach) (3×10⁻⁸ and 10⁻⁷ M) and papaverine (PAPA) (10⁻⁵ and 3×10⁻⁵ M). Calcium concentration in normal PSS is 1.6 mM. NE, norepinephrine. *Significantly different from 100%.

A reduction in Na⁺ alone to 80% of control levels had no influence on the dilation to acetylcholine.⁸

Cumulative acetylcholine relaxation concentration curves for rabbit ear resistance arteries expressed in relation to the equilibrium contraction to norepinephrine (10⁻⁶ M) were determined for single and combined reductions in Na⁺ and Ca²⁺ to 119 and 1.28 mM, respectively (Figure 4). These are 80% of normal levels in PSS. The reduction of Na⁺ to 119 mM did not influence the response to acetylcholine. The shift in the acetylcholine relaxation curve to the right caused by Ca²⁺ reduction to 1.28 mM alone was not changed by the simultaneous reduction in Na⁺ to 119 mM. Mean values for acetylcholine-induced relaxation over the entire concentration range were numerically smaller but did not reach a level of significance.

Increases in wall force to norepinephrine (10⁻⁶ M) (Figure 4) and relaxation to papaverine (1 and 3×10⁻⁵ M) were uninfluenced by these changes in Na⁺ and Ca²⁺.

Reversal of the Depression of Flow Dilation Resulting From a Decrease in Na⁺ by Subsequent Lowering of Ca²⁺.

Confirmation was sought of the above finding that, although a lowering of Na⁺ or of Ca²⁺ alone reduced flow dilation, lowering of both ions together had little or no effect; therefore, the consequence of Na⁺ reduction alone followed by the additional reduction of Ca²⁺ was observed. Figure 5 shows the effects on flow dilation of lowering Na⁺ alone followed in the same segment by a...
proportionate lowering of Ca$^{2+}$. Reductions were made of Na$^+$ by 5%, 10%, or 20% or to values ranging from 54% to 0% of control. In each experiment, the effect of the subsequent additional and proportionate lowering of Ca$^{2+}$ was to reverse the inhibitory effect on the dilator response of lowered Na$^+$. This increase, expressed as a percent of the control flow dilation in each case, ranged from approximately 40% to more than 100%.

These changes in ion concentrations had no effect on the contraction to norepinephrine (10$^{-8}$ M).

**Lack of Reversal of the Decrease of Flow Dilation Caused by Decrease in Na$^+$ by a Concomitant Increase in Ca$^{2+}$**

To show that the reversal of the depression of flow dilation caused by reduction in Na$^+$ that occurred when both Na$^+$ and Ca$^{2+}$ were proportionately reduced was not seen with the combination of other mechanisms that alone depressed flow dilation, we experimentally assessed the effect of combined Na$^+$ reduction and Ca$^{2+}$ increase in flow dilation. Reduction of Na$^+$ to 80% of normal value reduced flow dilation to 37.3±7.9% of control. Increase in Ca$^{2+}$ to 200% of its normal level, i.e., to 3.2 mM, resulted in a reduction in flow dilation to 21.4±10.7% of control (n=5). Concomitant reduction of Na$^+$ and increase in Ca$^{2+}$ resulted in almost complete obliteration of the dilator response (2.5±2.5% of control, n=3). Thus, the combination of these two initial conditions, both of which caused very significant reductions in flow dilation, resulted in an even greater reduction of the response. This suggested the uniqueness of the effect of the concomitant reduction of Na$^+$ and Ca$^{2+}$ on the flow-induced response.

**Discussion**

The new experimental findings in this study of the Na$^+$ and Ca$^{2+}$ dependence of flow dilation are as follows: 1) The decrease and also increase in Ca$^{2+}$ from its normal level in the PSS of 1.6 mM resulted in a reduction in flow dilation. The relaxation to acetylcholine diminished only with Ca$^{2+}$ decrease. The optimum Ca$^{2+}$ concentrations for flow dilation fell within the range of 1.44 to 1.92 mM. 2) The reduction in flow dilation caused by a decrease in Ca$^{2+}$ concentration was prevented by a proportionate decrease in Na$^+$. Na$^+$ reduction alone caused a decrease in flow dilation. This effect is true whether both ions were reduced concurrently before testing for flow dilation or whether the consequences of Na$^+$ reduction on flow dilation are first recorded and then the effect of additional Ca$^{2+}$ reduction was studied. The depression of acetylcholine dilation with reduction in Ca$^{2+}$ was not reversed by concomitant reduction in Na$^+$. 3) The reduction in flow dilation caused by the increase in Ca$^{2+}$ concentration was augmented by the concurrent reduction in Na$^+$. This finding emphasized the uniqueness of the interaction between Na$^+$ and Ca$^{2+}$ in relation to flow dilation. None of these ionic changes influenced the contraction of the artery strips to norepinephrine or their relaxation to papaverine. Thus, it is clear that flow-induced changes depend on different ionic mechanisms than do the responses of either of these pharmacological agents.

Both flow and acetylcholine dilation diminished by comparable amounts when extracellular Ca$^{2+}$ was reduced by 20%. Lopez-Jaramillo et al. concluded that...
NO production in the rabbit thoracic aorta in response to acetylcholine increased as Ca$^{2+}$ levels were elevated up to 1.25 mM and then decreased as Ca$^{2+}$ levels were further increased. The peak of the acetylcholine dilation response in this present study occurred at a higher Ca$^{2+}$ level than the peak for NO production but cannot be distinguished from that of flow in the same artery (Figure 1). Endothelial NO synthase is a Ca$^{2+}$-dependent enzyme, and presumably the Ca$^{2+}$ sensitivity of the acetylcholine dilation reflects this. A sizable component of flow-dependent dilation may be associated with an endothelium-derived relaxing factor–like substance released presumably from muscle cells. The nature of this proposed substance, the synthetic pathway involved, and its Ca$^{2+}$ sensitivity are unknown.

The size of the resistance artery contraction to noradrenaline was uninfluenced by either an increase or decrease in extracellular Ca$^{2+}$. This is consistent with previous observations. K$^+$(40 mM) and phenylephrine (10 μM)-induced tone of arterioles of the hamster cheek pouch did not change their diameter when the local Ca$^{2+}$ concentration was altered over this range. The tone of the thoracic aorta did not vary when Ca$^{2+}$ concentrations were varied over a considerably greater range. There was little variation in the magnitude of the contraction of helical strips of the rat tail artery to methoxamine with Ca$^{2+}$ changes between 1.6 and 4.1 mM. These observations suggest that vascular smooth muscle intracellular activator calcium concentration on exposure to a variety of agonists is independent of the variation in extracellular Ca$^{2+}$ level used in this study. This conclusion is consistent with the observation of Palant et al. on rat thoracic aorta; they failed to find a change in intracellular Ca$^{2+}$ using the dye fura 2-AM when extracellular Ca$^{2+}$ was reduced to 0.25 mM. Over the same range of calcium concentration, the membrane potential of the vascular smooth muscle from the common carotid artery does not vary significantly. The action of NO on blood vessel tone is uninfluenced by change in extracellular Ca$^{2+}$. These findings suggest that the effects of lowering Ca$^{2+}$ on the flow and dilation response to acetylcholine are not associated with lowering of intracellular Ca$^{2+}$. This conclusion, however, is not consistent with the idea that the sensitivity of acetylcholine dilation to lowered calcium reflects the diminished activity of a Ca$^{2+}$-sensitive intracellular enzyme.

A number of membrane-related mechanisms (for summary, see References 26 and 27) decrease with the increase in extracellular Ca$^{2+}$. Bohr has used the term “membrane stabilization” to describe these effects. However, they have been previously observed only in relation to higher extracellular Ca$^{2+}$ levels than those explored in these present series of experiments. A number of explanations of this “stabilizing” effect have been elaborated. Webb and Bohr proposed that the reduced response of vascular strips to norepinephrine was due to increased activity of the Na$^+$,K$^+$-ATPase. Furspan et al. introduced the idea that “membrane-bound calcium” reinforces the stabilizing effect of an electrical field on the resting membrane, a concept subsequently supported in part by Palant et al. It has been suggested that membrane stabilization is associated with Ca$^{2+}$ binding by an integral membrane Ca$^{2+}$ binding protein that presumably stabilizes a variety of membrane-related mechanisms. Lopez-Jaramillo et al. did not advance an explanation for the reduction they observed in NO production in high extracellular Ca$^{2+}$.

The similar sensitivity of flow constriction and dilation to a lowering of Na$^+$ leads to the proposal that the ion-sensitive mechanism must be at a site common to both flow effects. Possibilities include the flow sensor itself, a mechanism that couples this to the responsive cell, or both.

It has been proposed that the flow-sensitive system may reside in the extracellular matrix, possibly the glycosaminoglycans, some of which are known to span the cell membrane. Siegel and colleagues have studied the static binding properties of cations with glycosaminoglycan polyanions such as chondroitin-4-SO$_4$-polypeptide complex and heparan sulfate using nuclear magnetic resonance techniques. They found evidence consistent with competition between Na$^+$ and Ca$^{2+}$ for binding sites with Ca$^{2+}$ concentrations up to 0.3 mM. Over a higher concentration range, up to 3 mM, there was evidence for cooperative interaction between the two ions. They argue that this is due to a conformational change in the macromolecular binding of these ions resulting from the induction of intra- and intermolecular linking. At Ca$^{2+}$ concentrations higher than 3.0 mM, cooperativity is lost, and interaction between the two ions once more becomes competitive. The finding of this present study, that the ratio of Na$^+$ and Ca$^{2+}$ concentrations is important for flow dilation, is consistent with a cooperative binding of these two ions to the flow-sensing mechanism. It is also consistent with the idea (see above) that some of these effects of changing ions may reflect changes on the cell surface.

The additive effect on flow dilation of Ca$^{2+}$ increase and Na$^+$ decrease is consistent with the well-known cell membrane stabilizing effect of increased Ca$^{2+}$ concentrations (see above). Na$^+$ influx, which has been proposed as a possible intermediate mechanism for flow dilation, has been reported to be depressed by increasing external Ca$^{2+}$ concentration.

Alternative explanations of these experimental findings should be considered. Ca$^{2+}$ is known to regulate Na$^+$-K$^+$ (Cl) cotransport. However, bumetamide has no effect on flow dilation (Bevan and Joyce, unpublished observations). Ca$^{2+}$ is known to modify the activity of the Na$^+$ pump, but flow dilation is unaffected by 30-minute exposure to ouabain. Na$^+$/Ca$^{2+}$ exchange activity is generally considered to be low in vascular smooth muscle and vascular tone only influenced by larger changes in PSS Na$^+$ than those used in these present experiments. Ca$^{2+}$ may influence intracellular Na$^+$ secondary to its exchange with H$. However, the changes in extracellular Na$^+$ used in these studies were small in comparison to those necessary to activate the Na$^+$/H$^+$ exchange mechanism. There is little evidence that this exchange system significantly influences vascular tone.

The contribution of flow-induced changes in tone to circulatory homeostasis is not known. However, the magnitude of the flow-induced change in both large and small arteries and in veins and the observation that it can elicit both constriction and relaxation suggest that it may play a role in setting the level of basal tone in resistance arteries. These present experiments raise the
Possibility that vascular tone may be related to the relative as well as the absolute concentrations of extracellular Na\(^+\) and Ca\(^{2+}\).

**References**

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