Role of calcium in contractile responses of calf cardiac vein during cooling

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Abstract
The effects of cooling (to 28°C) were studied on the responses induced by carbachol (10⁻⁹ – 3 × 10⁻⁴ M) and serotonin (5-HT, 10⁻⁸ – 3 × 10⁻⁴ M) in calf cardiac vein preparations and the role of calcium ions in these effects were analyzed. Ring preparations of veins obtained from calf hearts were suspended in organ baths containing 25 mL of Krebs-Henseleit solution, maintained at 37°C and continuously gassed with 95%O₂–5%CO₂. After a resting period, preparations were contracted with carbachol (10⁻⁹ – 3 × 10⁻⁴ M) and 5-HT (10⁻⁸ – 3 × 10⁻⁴ M) at 37°C. The same protocol was repeated at 28°C after the preparations were allowed to equilibrate at this temperature for 60 min. In order to analyze the role of calcium ions (Ca²⁺) in the cooling-induced vascular response, concentration-response curves to carbachol and 5-HT were obtained in the presence of verapamil (10⁻⁶ M), caffeine (3 × 10⁻⁴ M), and Ca²⁺ free medium in the presence of EGTA at 28°C. During cooling to 28°C, the EC₅₀ values, to carbachol and 5-HT were significantly higher than at 37°C. Cooling to 28°C in the presence of verapamil, caffeine or Ca²⁺ free medium in the presence of EGTA increased the EC₅₀ values, to both carbachol and 5-HT. These results suggest that Ca²⁺ plays an essential role in the cooling-induced changes of calf cardiac vein preparations treated with carbachol and 5-HT.

Key words: calcium, carbachol, cardiac vein, cooling, serotonin

Introduction
Contractions of vascular smooth muscle caused by different agents are modulated by several factors, including temperature (Atalık et al., 2000; Atalık et al., 2007; Atalık et al., 2008). Experimental evidence shows that cooling affects the reactivity of vessels (Harker et al., 1991; Garcia-Villalon et al., 1992). Most of the previous studies examining the effect of temperature on smooth muscle responses to vasoactive agents have focused on cutaneous vessels, with information concerning noncutaneous vessels such as the cardiac vein being rather limited.

Calcium ion (Ca²⁺) plays a major role in the regulation of many cell functions. This ion makes its entrance into the cytoplasm either from outside the cell through the cell membrane via calcium channels, or from internal calcium storages. In our study, verapamil, caffeine and Ca²⁺-free...
medium with EGTA were used to determine if Ca\textsuperscript{2+} ions played a functional role in the cooling induced responses in calf cardiac vein. Verapamil is one of the most representative calcium channel blockers and acts as a blocker of L-type calcium channels in a voltage dependent manner (Romero et al., 2003). Caffeine is known to induce Ca\textsuperscript{2+} release from intracellular stores in muscular tissues (Karaki and Nagase, 1987; Van Breemen and Saida, 1989).

The endothelium appears to play a major role in the regulation of vascular tone by releasing relaxing and contracting factors (Furchgott and Vanhoutte, 1989). The endothelium can produce nitric oxide from L-arginine which causes vascular relaxation (Palmer et al., 1988). In addition to agonist-mediated constriction, limited evidence suggests that vascular smooth muscle responsiveness to nitric oxide and related factors can be influenced by temperature (Karaki and Nagase, 1987; Monge et al., 1993; Garcia-Villalon et al., 1995). Recently, we studied the effects of temperature on the contractile responses of smooth muscle preparations in a variety of vessels from different species and observed that the contractile responses were temperature-dependent, with the endothelium appearing to have no role in these responses (Atalik et al., 2000; Atalik et al., 2001a; Atalik et al., 2003).

The aim of the present study was to determine the effect of calcium on the responses of endothelium-denuded calf cardiac vein preparations during cooling. For this study, the calf cardiac vein was selected because it is easily accessible. Therefore, verapamil, caffeine, and a Ca\textsuperscript{2+}-free medium with EGTA were used in preparations constricted with carbachol and 5-HT during cooling.

**Materials and Methods**

**Tissue preparations**

Calf hearts were obtained from a slaughterhouse and were immediately placed in Krebs-Henseleit solution. Segments of the great cardiac vein were removed and cut into rings 2.5 mm in length. The endothelial layer was mechanically removed from the vascular rings. Each ring was mounted in a 25 mL organ bath containing Krebs-Henseleit Solution (KHS), aerated with 95% O\textsubscript{2} and 5% CO\textsubscript{2}. The composition of the KHS in mM was: NaCl 119, KCl 4.70, MgSO\textsubscript{4} 1.50, KH\textsubscript{2}PO\textsubscript{4} 1.20, CaCl\textsubscript{2} 2.50, NaHCO\textsubscript{3} 25, glucose 11.

Changes in isometric tension were recorded using a force-displacement transducer (BIOPAC MP36, Santa Barbara, California, USA) connected through amplifiers to an ITBS08 Integrated Tissue Bath System (Commat, Ankara, Turkey). The tissues were allowed to equilibrate for 60 mins under a resting tension of 1 g with repeated washing every 15 min.

**Experimental procedure**

First, cumulative concentration-response curves were determined in calf cardiac vein preparations for carbachol (10\textsuperscript{-9} – 3 \times 10\textsuperscript{-4} M) and 5-HT (10\textsuperscript{-8} – 3 \times 10\textsuperscript{-4} M) at 37°C. Then, another set of experiments was designed to determine the effect of cooling on the carbachol and 5-HT-induced contractile responses. After the first concentration-response curve was completed, preparations were washed and allowed to reestablish resting tension before being cooled. When preparations stabilized (30 min), the bath temperature was decreased to 28°C. Preparations were allowed to equilibrate at this temperature for 1 h before a second concentration-response curve
was determined. In order to analyse the role of Ca\(^{2+}\) in the cooling induced vascular response, concentration-response curves to carbachol and 5-HT were obtained in the presence of verapamil (10\(^{-6}\) M). Verapamil was added to the organ bath 20 min before concentration-response curves were obtained.

In another series of experiments, the tissues were preincubated with caffeine (3 × 10\(^{-4}\) M) to determine the role of intracellular calcium during cooling. The preparations were incubated for 20 min with caffeine at 28°C.

In order to analyze the role of Ca\(^{2+}\) in the cooling induced vascular response, concentration-response curves to carbachol and 5-HT were obtained in a Ca\(^{2+}\)-free medium. Preparations were equilibrated in Ca\(^{2+}\)-free solution for 30 min.

Parallel experiments were conducted in separate groups of preparations maintained at either 37°C or 28°C for the duration of the experiment to control for changes in responses due to time. Only one agent was tested in each preparation.

**Statistical analysis**

Concentrations of the contractile agents causing 50% of the maximal response (EC\(_{50}\)) were measured from each individual concentration-response curve. The maximum effect values were calculated as the percentage of the maximum response of the tissue to carbachol and KCl at 37°C and 28°C, respectively. Maximal responses and EC\(_{50}\) values for curves obtained before (control) and during cooling and during cooling (control II) and in the presence of verapamil or caffeine or in the Ca\(^{2+}\)-free medium during cooling were compared by using paired and unpaired Student’s t-test. Statistical significance was set at \(P<0.05\).

**Drugs**

Carbachol chloride, 5-HT, verapamil and caffeine were used. All drugs were obtained from Sigma, St. Louis, MO, USA.

**Results**

**Carbachol-induced contractions**

Figure 1 shows the effects of carbachol (10\(^{-8}\) – 3 × 10\(^{-4}\) M) on calf cardiac vein rings at both 37°C and 28°C after cooling, and also at 28°C in the presence of caffeine (3 × 10\(^{-4}\) M), verapamil (10\(^{-6}\) M), and Ca\(^{2+}\)-free medium with EGTA. At 37°C, carbachol produced concentration-dependent contractions (Table 1).

At 28°C, the sensitivity of cardiac vein preparations was significantly lower (9 times; \(P<0.05\)) than at 37°C (Table 1).

Treatment with caffeine (3 × 10\(^{-4}\) M) significantly decreased the sensitivity to carbachol during cooling (1.6 times; \(P<0.05\)) (Fig. 1, Table 1). At 28°C, the sensitivity to carbachol was significantly lower in the presence of verapamil (2.7 times; \(P<0.05\)) and also in Ca\(^{2+}\)-free solution (2.7 times; \(P<0.05\)) (Table 1).

There was no significant difference in the maximum responses to carbachol in the presence of each one of the agents used or in Ca\(^{2+}\)-free medium (Table 2).
Serotonin-induced contractions

Figure 2 shows the effects of 5-HT (10⁻⁸ – 3 × 10⁻⁴ M) on calf cardiac vein rings at both 37°C and 28°C after cooling, and also at 28°C in the presence of caffeine (3 × 10⁻⁴ M), verapamil (10⁻⁶ M), and also Ca²⁺ free medium with EGTA at 28°C.

Each point is the mean ± SEM of 6 experiments.

Table 1. EC₅₀ values for carbachol and 5-HT in calf cardiac vein preparations at both 37°C and 28°C, in the presence of caffeine, verapamil and also in Ca²⁺-free medium at 28°C

|          | EC₅₀ (× 10⁻⁶ M) Carbachol | EC₅₀ (× 10⁻⁵ M) 5-HT |
|----------|---------------------------|----------------------|
| 37°C (n=6) | 3.7 ± 0.3                 | 2.2 ± 0.1             |
| 28°C (n=6) | 32.7 ± 0.1*               | 6.0 ± 0.3*            |
| 28°C, Caffeine (n=6) | 52.6 ± 1.8**             | 8.0 ± 0.1**           |
| 28°C, Verapamil (n=6) | 86.7 ± 1.5**              | 10.0 ± 0.3**          |
| 28°C, Ca²⁺ free (n=6) | 89.3 ± 1.6**              | 12.0 ± 0.2**          |

Each value is derived from 6 experiments. Values are given as mean ± SEM of six experiments. *, P<0.05 compared to EC₅₀ values obtained at 37°C. **, P<0.05 compared to EC₅₀ values obtained at 28°C.

Table 2. Maximum responses of calf vein preparations to carbachol and 5-HT at both 37°C (control) and at 28°C, in the presence of verapamil, caffeine, and Ca²⁺-free medium with EGTA

|          | Eₘ₅₀ Carbachol 5-HT |
|----------|---------------------|
| Control  | 100 ± 0.0           | 100 ± 0.0           |
| Verapamil| 99 ± 0.8            | 99 ± 1.0            |
| Caffeine | 99 ± 0.6            | 99 ± 0.7            |
| Ca²⁺-free EGTA | 98 ± 1.1    | 98 ± 0.8            |

Values are given as mean ± SEM of six experiments.

Serotonin-induced contractions

Figure 2 shows the effects of 5-HT (10⁻⁸ – 3 × 10⁻⁴ M) on calf cardiac vein rings at both 37°C and 28°C after cooling, and also at 28°C in the presence of caffeine (3 × 10⁻⁴ M), verapamil (10⁻⁶ M), and also Ca²⁺ free medium with EGTA at 28°C.
At 37°C, 5-HT produced concentration-dependent contractions (Table 1). At 28°C, the sensitivity of the cardiac vein preparations to 5-HT was significantly lower (2.7 times; \( P < 0.05 \)) than at 37°C (Table 1).

Treatment with caffeine (3 × 10^{-4} M) significantly decreased the sensitivity to 5-HT during cooling (1.3 times; \( P < 0.05 \)) (Fig. 2, Table 1). At 28°C, the sensitivity to 5-HT was significantly lower in the presence of verapamil (1.7 times; \( P < 0.05 \)) and also in Ca^{2+}-free solution (2.0 times; \( P < 0.05 \)) (Table 1).

There was no significant difference in the maximum responses to 5-HT in the presence of each one of the agents used or in Ca^{2+}-free medium (Table 2).

**Discussion**

We have recently observed that cooling (to 28°C) induced subsensitivity to carbachol and 5-HT in calf coronary artery and cardiac vein preparations (Atalik et al., 2001a; Atalik et al., 2001b). In these studies, we have also observed that endothelial nitric oxide does not play a role in the decreased sensitivity of noncutaneous vessels to contractile agents during cooling. Our results in noncutaneous vessels agree with those of other authors (Garcia-Villalon et al., 1992; Fernandez et al., 1994). However, the mechanism underlying the inhibitory effect of cooling on noncutaneous vessels to contractile agents is not clear.

In the present study, we have investigated the role of Ca^{2+} ions on the carbachol and 5-HT-induced contractions in preparations of an endothelium-denuded noncutaneous vessel, such as the calf cardiac vein, during cooling. The results indicate that cooling decreased the sensitivity to both agents. The cardiac vein is an easily accessible noncutaneous blood vessel and the mechanism of
the cooling-induced effects on carbachol and 5-HT-induced contractions on an endothelium-denuded vessel such as this has not been studied before. The coronary venous system (cardiac veins) collects one-third of the blood of the coronary circulation and is considered to be an important site for the blood-tissue exchange of water and nutrients, as well as of possible determinants of ventricular distensibility.

The temperature of 28°C used for cooling in this study was considered to be a “moderate cooling” temperature as determined by our previous studies (Atalik et al., 2000; Atalik et al., 2001a; Atalik et al., 2001b; Atalik et al., 2007; Atalik et al., 2008; Atalik et al., 2011).

Our results indicate that at 37°C, carbachol and 5-HT both induced concentration-dependent contractions in calf cardiac vein preparations. Compared with the control responses at 37°C, cooling decreased the sensitivity, but not the maximal contraction, to both carbachol and 5-HT.

As we know, carbachol and 5-HT can cause contractions both via cellular and extracellular Ca²⁺ (Leal-Cardoso et al., 2002; Perez and Sanderson, 2005). A calcium channel blocker (verapamil), Ca²⁺-free medium and caffeine were used to determine whether Ca²⁺ ions played a functional role in the cooling induced decreased sensitivity to carbachol and 5-HT.

It is known that Ca²⁺ ions play an essential role in the contractions of vascular smooth muscle (Brading and Sneddon, 1980). Verapamil is pharmacologically classified as a Ca⁺⁺-channel blocker for its presumed common properties of being able to block calcium influx in cardiac and vascular smooth muscle (Schawartz and Triggle, 1984). Our data showed that during cooling the sensitivities to both carbachol and 5-HT were decreased by verapamil, suggesting that the mechanism activated by cooling was coupled to an extracellular influx of calcium through voltage-dependent L-type calcium channels present on the vascular smooth muscle cells (Fabi et al., 1993; Simonet et al., 2000).

In this study, both caffeine and Ca²⁺-free solution with EGTA were used to examine the dependence on intracellular and extracellular calcium of the cardiac smooth muscle responses during cooling. Both calcium release from and uptake into internal sarcoplasmic reticulum (SR) Ca²⁺ stores play a central role in the activity of most cells, including that of excitation-contraction coupling in smooth muscle. The rise in free cytoplasmic Ca²⁺ level that is necessary for vascular smooth muscle contraction to occur can also be brought about by activation of the ryanodine receptor located on the SR by a spike in cytoplasmic Ca²⁺; the calcium-induced calcium release mechanism (Itoh et al., 1985; Gregoire et al., 1993). These receptors are sensitive to caffeine for ryanodine (Ehrlich et al., 1994). In this study, the effect of cooling on Ca²⁺ release from intracellular stores sensitive to caffeine was analyzed, wherein it was found that caffeine did not increase the sensitivity to carbachol and 5-HT during cooling. Moreover, cooling to 28°C after treatment with caffeine decreased the sensitivity to both carbachol and 5-HT. Here, we observed that intracellular calcium release did not increase the sensitivity to carbachol and 5-HT during cooling. Furthermore, Ca²⁺-free solutions with EGTA can be used to define the function of intracellular calcium (Herrera et al., 2000; Burt, 2008). In our study, incubation in Ca²⁺-free medium with EGTA decreased the sensitivity to both agents.

In conclusion, the mechanism of the effects of cooling in noncutaneous vessels is uncertain, but the finding in this study suggests a role for Ca²⁺ in the cooling-induced changes.
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