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European Journal of Pharmacology 362 (1998) 157–166

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# Endothelium-mediated negative dromotropic effects of intravascular acetylcholine

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Received 13 October 1998; accepted 16 October 1998

**Abstract**

Acetylcholine acting through specific muscarinic membrane receptors causes a negative dromotropic effect and, in blood vessels, causes a vasodilation which results from its action on the endothelial cells via release of nitric oxide (NO). We decided to study this effect in isolated Krebs–Henseleit retrogradely perfused guinea pig hearts. A pair of stimulating electrodes was placed in the right atrium and to record the auricular–ventricular interval (A–V delay) one recording electrode was placed on the left atrium and the other on the tip of the ventricle. Hearts were paced at a rate of $3.8 \pm 0.1$ Hz and perfused at a coronary flow rate of $9 \pm 0.25$ ml/min. To obtain dose–response curves, single doses (as boluses) of acetylcholine were infused and the maximal A–V delay induced by each dose was determined. Perfusion of agents that inhibit NO accumulation (L-Arginine methyl ester (L-NAME) (0.5 mM)) or oxyhemoglobin (6 μM) caused displacement of the acetylcholine dose–response curve downward and to the right. Perfusion of NO-sparing agents like superoxide dismutase and dithiothreitol caused an upward and leftward displacement. Infusion of NO solutions or a NO donor (diethylamine–nitric oxide [DEA–NO]) caused a dose-dependent negative dromotropic effect. In contrast, inhibition of the prostaglandin metabolic pathway by Indomethacin (0.01 mM) caused potentiation of acetylcholine effects which were reversed when it was co-perfused with L-NAME. When endothelial intravascular muscarinic receptors were selectively blocked by perfusion of a non-permeable macromolecule: dextran (> 2000 kDa) covalently complexed to the receptor blocker (3-(2′-aminobenzhydryloxy) tropane)), the negative dromotropic effect of intravascular acetylcholine was diminished in a concentration-dependent manner up to complete blockade. Our data indicate that the dromotropic effect caused by intracoronary administration of acetylcholine is the result solely of activation of intravascular endothelial muscarinic receptors, that nitric oxide and prostaglandins are non-synergistic endothelial mediators of this effect and that there may be an interaction between NO and prostaglandin metabolic pathways. 

*Keywords:* Luminal endothelial receptor; Endothelial control; Parenchymal function; Endothelial mediator; Nitric oxide (NO); Prostaglandin; Cardiac control

## 1. Introduction

Acetylcholine acting through specific muscarinic membrane receptors (Endoh et al., 1985; Hulme et al., 1990; Cauldfield, 1993; Bruning et al., 1994) in endothelium induces vasodilation (Cauldfield, 1993) and in the various cardiac muscle tissues, a decrease in sinoatrial node rate of discharge, decrease in auricular–ventricular node conduction rate and an auricular and ventricular negative inotropic effect (Alanis et al., 1958, 1959; Diamond et al., 1977; Endoh et al., 1985; Löffelholz and Pappano, 1985; Komori et al., 1988; Balligand et al., 1993; Cauldfield, 1993). Acetylcholine in intact animals is released at vagal endings and, experimentally added to isolated blood vessels in a tissue bath or injected intravascularly into experimental animals initiates endothelium-dependent blood vessel relaxation (Furchgott et al., 1987; Amezcua et al., 1988).

Exogenously applied acetylcholine induces its vasodilator effects by acting on the endothelial cells stimulating the synthesis and release of endothelium-derived relaxing factor (EDRF) or nitric oxide (NO). NO diffuses to adjacent

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0014-2999/98/$ - see front matter 
PII: S0014-2999(98)00755-9

smooth muscle cells, leading to relaxation. If the endothelium is injured, acetylcholine causes vasoconstriction, showing the important role of the endothelial lining in effects attributed to acetylcholine. The duration of acetylcholine action is brief due to its rapid metabolism by local and circulating acetylcholinesterase. These results suggest that the acetylcholine negative dromotropic effects may also result from endothelium-mediated mechanisms such as NO release. Evidence indicates that NO may cause its different cardiac effects through an increase in intracellular levels of cyclic guanosine monophosphate (cGMP) (George et al., 1970; Fink et al., 1976; Diamond et al., 1977; Metsa-Ketela et al., 1980; Wahler and Sperelakis, 1985; Goyal, 1989; Balligand et al., 1993). However, whether cGMP is involved in the negative dromotropic effect of acetylcholine has not been completely explored (George et al., 1970; Goyal, 1989).

The purpose of this study was to define the participation of the endothelium and of endothelial mediators in the negative dromotropic effects of intracoronary administration of acetylcholine and to establish that the selective blockade of intravascular muscarinic endothelial receptors prevents the negative dromotropic effects of intravascular acetylcholine.

Our results with isolated perfused guinea pig heart showed that (1) intracoronary administration of acetylcholine causes a negative dromotropic effect through activation of intravascularly located endothelial receptors, (2) this effect is mediated by the release of at least two mediators: NO and an arachidonic acid metabolism derivative.

## 2. Materials and methods

### 2.1. Isolated saline-perfused hearts

Male guinea pigs (350–450 g) were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and heparin sodium (500 U). The animals were artificially ventilated, the chests were opened, and a loose ligature was passed through the ascending aorta. The hearts were rapidly removed, immersed in ice-cold physiological saline, retrogradely perfused via a non-recirculating perfusion system at constant flow, and trimmed of non-cardiac tissue. Coronary flow was adjusted with a variable speed peristaltic pump (Harvard Apparatus, model 1215). An initial perfusion rate of 25 ml/min for 5 min was followed by a 25-min equilibration period at a perfusion rate of 10 ml/min. After this period of equilibration, the experiment was started and all hearts were perfused at a constant coronary flow rate of $9 \pm 0.25$ ml/min. The perfusion medium was Krebs–Henseleit solution (K–H) with the following composition (mM): 117.8 NaCl, 6 KCl, 1.75 $CaCl_2$, 1.2 $MgSO_4$, 1.2 $NaH_2PO_4$, 24.2 $NaHCO_3$, 5 glucose and 5 sodium pyruvate. The solution was equilibrated with 95% $O_2$–5% $CO_2$ (37°C at pH = 7.4).

### 2.2. Electrical stimulation and recording procedures

Recording and stimulating electrodes consisted of small stainless-steel wire vascular clamps (Fine Surgical Instruments) soldered to a thin flexible wire. The clamps were fixed to the myocardial surface by gently clamping the epicardial tissue layer. For each heart, care was taken to apply the recording and stimulating electrodes at the same sites in the cardiac chambers. A pair of stimulating electrodes was placed 2 mm apart in the apex of the right atrial appendage. Pacing was achieved by applying electrical square pulses of 2 ms duration and twice the electrical threshold at a rate of $3.8 \pm 0.1$ Hz. To record the auricular and ventricular electrocardiogram, one electrode was placed in the left atrium and a second electrode on the apex of the left ventricle, and these two electrodes were connected to an oscilloscope synchronized with the atrial pacing stimulator. The interval between application of the stimulus to the atrium and the rising phase of the atrial electrogram was $18.5 \pm 0.8$ ms and remained constant under control conditions and throughout the various pharmacological manipulations. To measure changes in the delay of the auricular–ventricular nodal area, we measured changes in the interval between atrial and ventricular electrograms (A–V delay) because of its simplicity and because we (Rubio et al., 1995) and others (Alanis et al., 1958, 1959; Clemo, 1986; Belardinelli et al., 1987) have shown that, under a variety of experimental conditions, changes in A–V delay are due solely to changes in the delay on the auricular–ventricular nodal area and not to the delays from atrium to node or from His blundle to ventricle, both of which remain constant. The auricular–ventricular delay (ms) was continuously monitored and measured as the interval between application of the stimulus and rising phase of the ventricular electrical signal. Under control conditions it had a mean value of $81 \pm 4$ ms.

Measurements of the auricular–ventricular delay during acetylcholine bolus experiments were done with the aid of a Polaroid camera mounted on the oscilloscope display panel. Manual opening of the shutter simultaneously with the acetylcholine bolus injection recorded on film the control A–V delay and its gradual increase for successive electrocardiographic tracings. For each dose of acetylcholine, the response amplitude was measured on the film as maximal increase in A–V delay with respect to the control.

### 2.3. Acetylcholine dose–response curves

Single doses of acetylcholine in the range of $6 \times 10^{-11}$ mol to $90 \times 10^{-11}$ mol were administered intracoronarily as boluses, either as variable volumes (1–30 μl range) of a

stock solution of acetylcholine ($3 \times 10^{-5}$ M), or as a constant 15-μl volume of solutions of different acetylcholine concentrations. In both cases independently of the volume of the bolus the injection period was constant and lasted 0.4 s. Bolus infusions were performed using a programmable infusion pump with a dispensing timer that runs the pump at preset rate and time of infusion (Harvard App. Model 55-222). Since the duration of the bolus infusion was constant, the doses of acetylcholine were expressed as moles and for each dose, the response amplitude was measured as the maximal increase in A–V delay with respect to the control. The results obtained with either procedure (variable or constant volume) of acetylcholine infusion were the same and their results were combined. In all experiments each heart was its own control. First, a control acetylcholine dose–response curve was obtained, followed by an acetylcholine dose–response curve (experimental) in the presence of $N^G$-nitro-L-arginine methyl ester (L-NAME, 0.5 mM), oxyhemoglobin [6 μM], superoxide dismutase (100 U/ml) or dithiothreitol (0.2 mM). Each group consisted of six hearts.

### 2.4. Oxyhemoglobin preparation

Oxyhemoglobin was prepared as described by Kelm and Schrader (1990). Briefly: hemoglobin was dissolved in distilled water (pH = 7), equilibrated with 100% oxygen, reduced with a molar excess of sodiumdithionite and purified by gel chromatography (Sephadex G25, Pharmacia). The purity of the eluted fraction was controlled spectrophotometrically.

### 2.5. Nitric oxide (NO) and diethylamine–nitric oxide (DEA–NO) solutions

In order to scavenge higher nitrogen oxides that may contaminate NO, the NO (Aldrich) was passed from its tank into a hermetically closed glass chamber bubbled through 100% nitrogen-equilibrated potassium hydroxide (10% wt./vol) solution. The NO from this chamber was passed into a second one and bubbled through nitrogen equilibrated distilled water to prepare a 100% saturated solution. Further dilutions of this solution were made with $N_2$-equilibrated distilled water. All solutions were collected in airtight syringes and infused into the coronary perfusion line as close as possible to the coronary entrance. A 1-mM solution of DEA–NO, a nitric oxide donor (Marangos, 1991), was infused intracoronarily at a rate to achieve a final 10-μM concentration. There were five hearts for each group.

### 2.6. Procedure for coupling 3-(2′-aminobenzhydryloxy) tropane (ABT) to high molecular weight ($2 \times 10^6$ Da) dextran

The procedure utilized was a modification of that published by Haga and Haga (1983, 1985). Briefly: 1 g of dextran ($5 \times 10^{-7}$ mol) was dissolved in 50 ml of distilled water and 1.2 g of $NaIO_4$ ($5.55 \times 10^{-3}$ mol) dissolved in 20 ml of water. These two solutions were mixed and the reaction was allowed to continue for 90 min with continuous stirring. For each glucose moiety of the dextran, this reaction caused oxidation of two adjacent carbons with hydroxyl groups, forming a pair of reactive aldehyde endings. The reaction was terminated by adding glycerol to a final concentration of 5 mM. To the periodate-oxidized dextran solution ice-cold methanol was gradually added in excess to cause precipitation. The suspension was centrifuged at $3000 \times g$ for 15 min and the pellet was resuspended in 50 ml of sodium phosphate buffer pH = 7; 1.5 g of 6-aminocaproic acid ($11 \times 10^{-3}$ mol) was added and the reaction was allowed to proceed 3 h with continuous stirring. In this reaction, the terminal amino group of 6-aminocaproic acid reacted with the aldehyde groups of the oxidized dextran forming a Schiff base which was reduced and stabilized on addition of 1 g of $NaCNBH_3$ and the reaction mixture was allowed to stand overnight at room temperature. The solution was dialyzed against several changes of distilled water, precipitated with methanol and dried. The product formed, dextran–6-aminocaproic acid, was dissolved in 50 ml of a mixture of dioxane and 0.1 M sodium phosphate buffer (30/70) and divided into two aliquots. One of the aliquots (control, solution 1) had no other addition made; to the other aliquot, 250 mg ($7.75 \times 10^{-4}$ mol) of 3-(2′-aminobenzhydryloxy) tropane dissolved in 5 ml dioxane was added with continuous stirring. This solution was further divided into two aliquots and 1 g of [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide] was added to only one of them, to activate the free carboxylate group of 6-aminocaproic acid to reach with the amino group of ABT, forming a dextran–6-aminocaproic acid–3-(2′-aminobenzhydryloxy)tropane complex. The purpose of the solution that did not receive the carbodiimide was to check for non-covalent binding of 3-(2′-aminobenzhydryloxy) tropane to the dextran–6-aminocaproic acid complex (control 2). The three different aliquots were allowed to stand for 3 h at room temperature. The products were dialyzed against several changes of water/dioxane (70/30). The complexes dextran–6-aminocaproic acid of each solution were precipitated in ice-cold acetone and extracted several times with benzene in order to eliminate any adsorbed 3-(2′-aminobenzhydryloxy) tropane. The UV absorption spectrum of an aqueous solution of dextran–6-aminocaproic acid–3-(2′-aminobenzhydryloxy) tropane (dextran–6AC–ABT) yielded an optical spectrum very similar to that of the ABT solution.

### 2.7. Acetylcholine-A–V delay dose–response curves in the presence of dextran–6-aminocaproic–3-(2′-aminobenzhydryloxy) tropane complex

A control acetylcholine dose–response curve was determined. Thereafter, an experimental curve was obtained in

the presence of either 0.025 μM or 0.25 μM dextran–6-aminocaproic acid–3-(2′-aminobenzhydryloxy) tropane or 0.25 μM of the two control solutions: controls 1 and 2. These experiments were designed to rule out any effect of the dextran–6-aminocaproic acid alone (control 1), non-covalent binding of 3-(2′-aminobenzhydryloxy) tropane to dextran–6-aminocaproic (control 2) and to explore the effects of the dextran–6-aminocaproic acid–3-(2′-aminobenzhydryloxy) tropane complex. In addition, to rule out the possibility of 3-(2′-aminobenzhydryloxy) tropane hydrolysis from the dextran–6-aminocaproic acid–3-(2′-aminobenzhydryloxy) tropane complex during its passage through the heart, venous effluents were collected during infusion of dextran–6AC–ABT. The venous effluents were extracted with benzene and the benzene was evaporated to dryness, the residue which could have contained free ABT was resuspended in a mixture of dioxane/Krebs–Henseleit (1/99) (control 3). A control dose–response to acetylcholine was performed with a second heart preparation, followed by another curve during infusion of solution control 3. There were six hearts per group.

### *2.8. Effect of indomethacin on acetylcholine dose–response curves*

In order to evaluate the role of arachidonic acid metabolic pathways on the activation of muscarinic receptors, control acetylcholine dose–response curves were performed with six hearts, thereafter the curve was repeated first in the presence of 0.01 mM indomethacin, and second in the presence of a mixture of indomethacin plus 0.5 mM L-NAME.

#### *2.8.1. Statistics*

Values are expressed as means ± SE. In these experiments, each heart was its own control, responses under control conditions and during specific manipulations were compared in the same heart. Statistical significance was determined using a paired *t*-test with a Bonferroni correction factor for multiple comparisons. Statistical significance was accepted for values of $P \leq 0.05$.

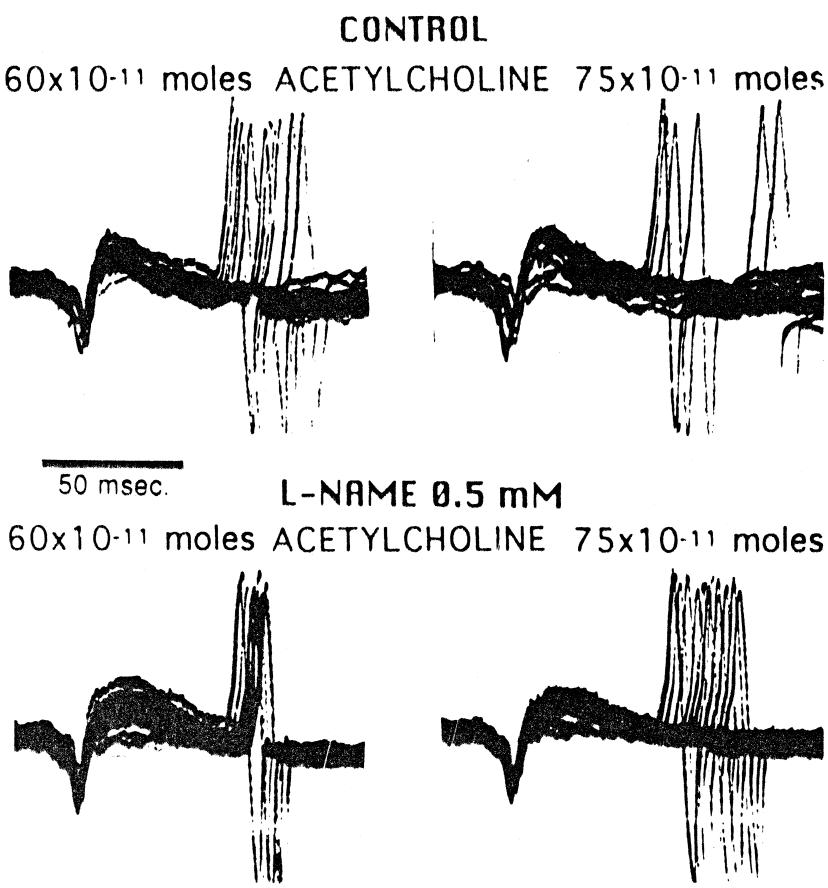


Fig. 1. Diminution of the negative dromotropic effect of acetylcholine by L-NAME. Auricular and ventricular electrocardiograph traces. The beginning of each trace marks the time the pacing stimulus was applied to the left atrium. Intracoronary single doses of $60 \times 10^{-11}$ mol and $75 \times 10^{-11}$ mol of acetylcholine in the absence (upper panels) and the presence of L-NAME (0.5 mM) (lower panels). Notice that for each dose of acetylcholine the ventricular delay for successive beats increased to reach a maximum and that these effects were depressed by L-NAME.

## 3. Results

### 3.1. Acetylcholine dromotropic effects. The role of nitric oxide

The application of intracoronary boluses of acetylcholine to perfused guinea pig hearts resulted in a dose-dependent transient negative dromotropic effect. Figs. 1 and 2 show representative electrograms of the effects of $60 \times 10^{-11}$ mol and $75 \times 10^{-11}$ mol (upper two traces) doses of acetylcholine. In each case, there was a gradual increase in the auricular–ventricular delay with each successive beat. In addition, there was a greater incremental increase with the larger dose. The control dose–response curves for acetylcholine are shown in Figs. 3 and 4. In the abscissae are the moles per bolus of acetylcholine and in the ordinates the differences between the maximal increase in A–V delay for each dose minus the A–V delay value prior to acetylcholine administration.

### 3.2. Effect of agents that inhibit nitric oxide accumulation: L-NAME and oxyhemoglobin

Administration of acetylcholine during either L-NAME (0.5 mM) or oxyhemoglobin (6 μM) perfusion, caused a smaller effect than under control conditions. Figs. 1 and 2 show how the negative dromotropic effects of acetylcholine in the presence of these inhibitors (lower traces) are depressed as compared to the corresponding controls (upper traces). Fig. 3A and B show that the acetylcholine dose–response curve during either L-NAME or oxyhemoglobin perfusion was displaced downward and to the right as compared with its control.

### 3.3. Effect of agents with a nitric oxide sparing effect: superoxide dismutase and dithiothreitol

Superoxide dismutase is an enzyme that inhibits the inactivation of nitric oxide by anion superoxide and dithiothreitol, an antioxidant substance; both protect nitric oxide from deactivation (Gryglewski and Palmer, 1986; Rubanyi and Vanhoutte, 1986). In Fig. 4A and B, it can be seen that the perfusion of 100 U/ml of both superoxide dismutase and 0.2 mM dithiothreitol potentiates the negative dromotropic effect of acetylcholine. Both agents caused an upward and leftward displacement of the acetylcholine-negative dromotropic effect curve.

### 3.4. Negative dromotropic effect of infusion of nitric oxide and the nitric oxide donor DEA–NO

The effects of continuous infusion of three different NO solutions with 70%, 80% and 100% saturation at a rate of

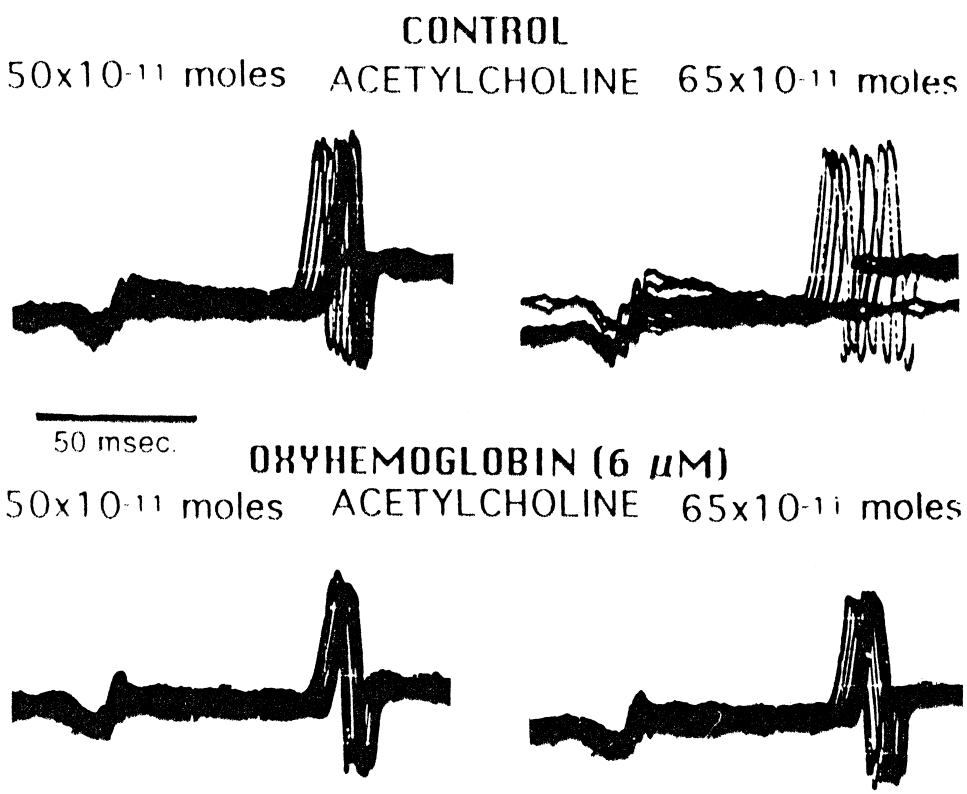


Fig. 2. Diminution of the negative dromotropic effect of acetylcholine by oxyhemoglobin. Auricular and ventricular electrocardiograph traces. The beginning of each trace marks the time the pacing stimulus was applied to the left atrium. Intracoronary doses of $50 \times 10^{-11}$ mol and $65 \times 10^{-11}$ mol of acetylcholine in the absence (upper panels) and presence of oxyhemoglobin (6 μM) (lower panels). Notice that for each bolus of acetylcholine the ventricular delay for the successive beats increased to reach a maximum and that these effects were depressed by oxyhemoglobin.

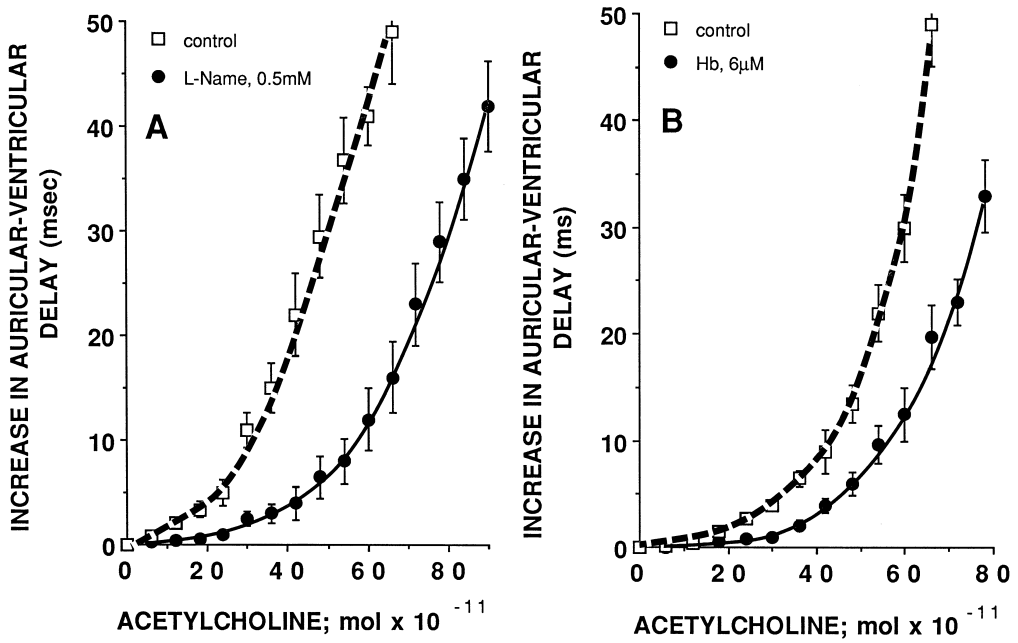


Fig. 3. Diminution of the negative dromotropic effect of acetylcholine by L-NAME (A) and oxyhemoglobin (B). Abscissae, boluses of acetylcholine (mol $\times 10^{-11}$). Ordinates, increase in A–V delay (ms, mean $\pm$ SE). Acetylcholine effects in the absence (open symbols) or in the presence of L-NAME (panel A) or oxyhemoglobin (panel B). In A as in B, the differences between control and experimental curves were significantly different $P < 0.05$.

0.12 ml/min are shown in Fig. 5A and the effects of continuous infusion of 10 μM DEA–NO are shown in Fig. 5B. There was a gradual increase in the A–V delay. Clearly, in the case of NO the rate of increase in transmission time was concentration-dependent being slower for 70%, faster for 80% and fastest for 100%. In the case of infusion of the 100% saturation NO solution, total A–V blockade was reached after 70 s of infusion.

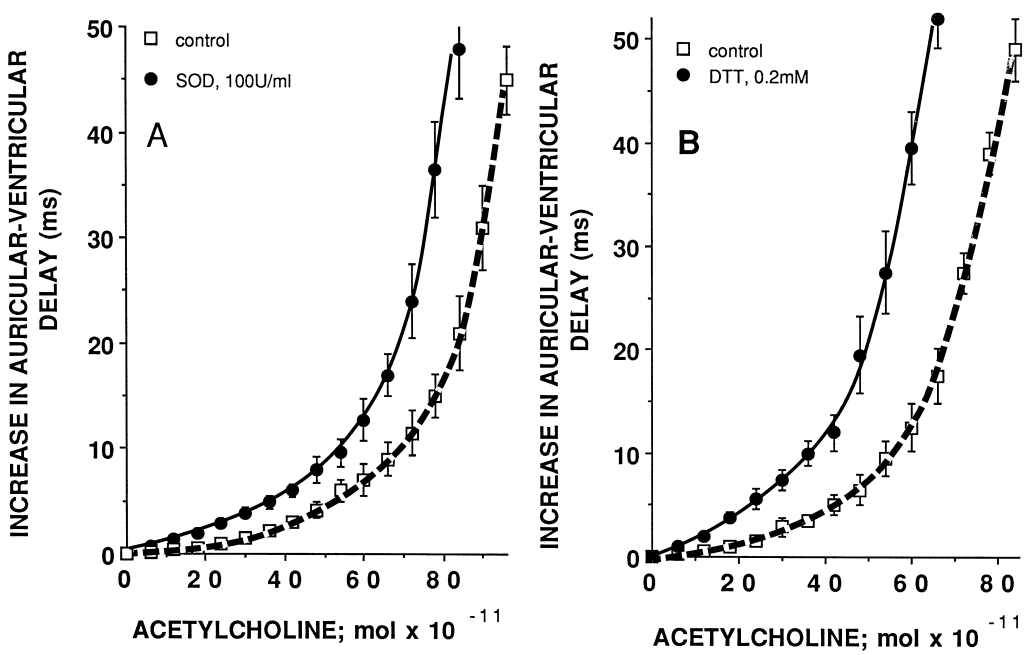


Fig. 4. Potentiation of the negative dromotropic effect of acetylcholine by superoxide dismutase (SOD, panel A) and dithiothreitol (DTT, panel B). Abscissae, boluses of acetylcholine (mol $\times 10^{-11}$). Ordinates, maximum increase in A–V delay (ms, mean $\pm$ SE). Acetylcholine effects in the absence (open symbols) or in the presence of SOD (panel A) or oxyhemoglobin (panel B). In panel A as in panel B, the differences between control and experimental curves were significantly different $P < 0.05$.

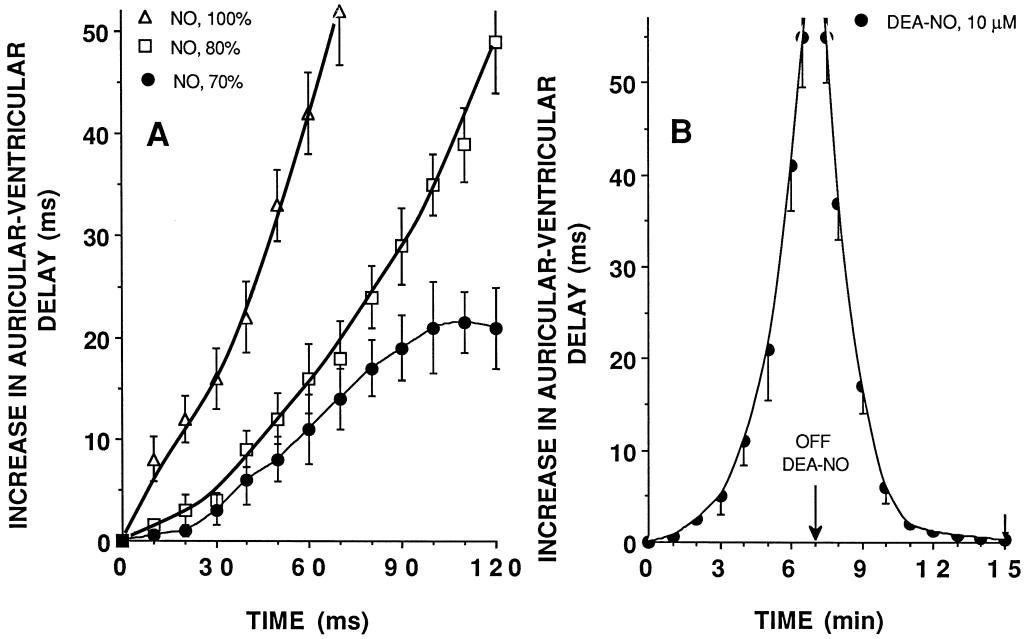


Fig. 5. Time course of increase in auricular–ventricular delay (mean ± SE) as a consequence of infusion of nitric oxide solutions (panel A) and the NO donor DEA–NO (panel B, 10 μM). In both panels, infusion of the agents was initiated at zero time. In A, concentrated solutions of NO—100, 80 and 70% —were infused at a rate of 0.12 ml/min the more concentrated being the most effective. In B, the second arrow marks the time DEA–NO was stopped. Notice the reversal of the effect.

In the case of the nitric oxide donor, DEA–NO, the gradual increase in A–V delay led to complete blockade at 6.5 min. Stopping the infusion of all of these agents caused a complete reversal of their effect.

### *3.5. Blockade of acetylcholine dromotropic effect by 2000 kDa dextran–6-aminocaproic–3-(2′-aminobenzhydryloxy) tropane complex*

The dose–response curve for acetylcholine was not modified when acetylcholine was administered either during infusion of 0.25 μM dextran–6-aminocaproic acid (control 1) or during infusion of dextran–6-aminocaproic acid preincubated with 3-(2′-aminobenzhydryloxy) tropane without the presence of carbodiimide (control 2) indicating that dextran–6-aminocaproic acid (control 1) was not active and that there was no non-covalent absorption of 3-(2′-aminobenzhydryloxy) tropane to dextran–6-aminocaproic acid (results not shown).

Our results showed that the perfusion of $>2000$ kDa dextran–6AC–ABT complex induced, in a dose-dependent manner, a downward and rightward shift of the acetylcholine-induced negative dromotropic effect curve (Fig. 6). Dextran–6AC–ABT, at a final concentration of 0.025 μM, caused a considerable degree of blockade of acetylcholine dromotropic effects and the blockade was complete with 0.25 μM dextran–6AC–ABT. At this concentration, in order to elicit a response it was necessary to increase the highest acetylcholine concentration by a factor of 100.

The control dose–response curve to acetylcholine was not modified when acetylcholine was added during infusion of control solution 3 (results not shown). This indicates that dextran–6AC–ABT is not hydrolyzed during its passage through the heart because the venous effluents of hearts perfused with dextran–6AC–ABT did not show the presence of free ABT.

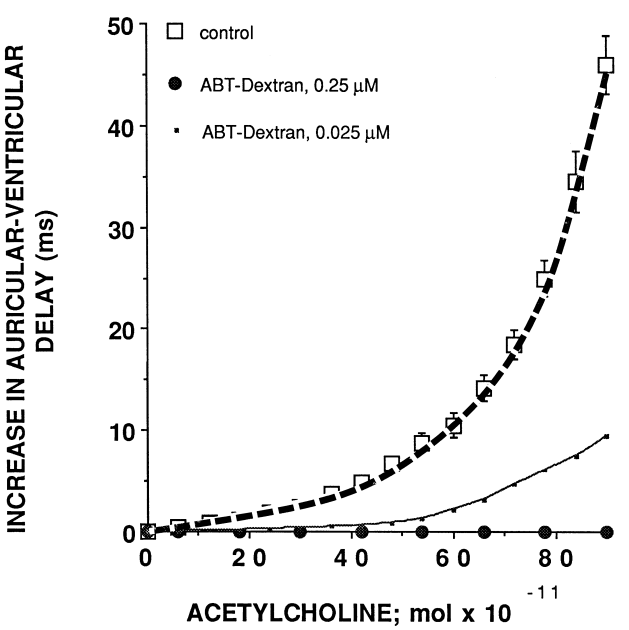


Fig. 6. Blockade of the acetylcholine negative dromotropic effect by macromolecular complex ABT–dextran. Abscissae, bolus of acetylcholine ($\text{mol}\times10^{-11}$). Ordinates, increase in A–V delay (ms). Squares, control; full circles, ABT-dextran 0.25 μM; and dots, ABT-dextran 0.025 μM. Curves are significantly different ($P<0.05$) from control conditions.

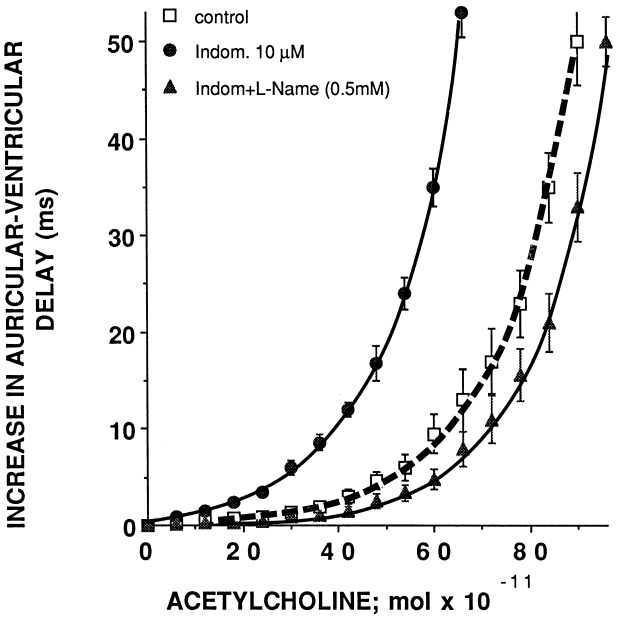


Fig. 7. Potentiation of the negative dromotropic effect of acetylcholine by indomethacin and reversal of this potentiating effect by L-NAME. Abscissae, boluses of acetylcholine (mol $\times 10^{-11}$). Ordinates, increase in A–V delay (ms, mean $\pm$ SE). Effects of acetylcholine alone (squares), in the presence of indomethacin (full circles) and in the presence of indomethacin plus L-NAME (triangles). Curve in the presence of indomethacin was significantly different ($P < 0.005$) from control. Curve in the presence of indomethacin plus L-NAME was significantly different ($P < 0.005$) from control only at doses of acetylcholine greater than $65 \times 10^{-11}$ mol.

### *3.6. Effect of indomethacin on acetylcholine dose–response curves*

The presence of indomethacin (0.01 mM) in the K–H solution potentiated the effects of acetylcholine. This indomethacin effect induced an upward and leftward shift of the acetylcholine negative dromotropic effect curve (Fig. 7). This potentiating effect of indomethacin was reversed when indomethacin was co-perfused with L-NAME (0.5 mM) (Fig. 7).

## 4. Discussion

Our data support the concept that the dromotropic effects caused by the intracoronary administration of acetylcholine are the result of activation of intravascular muscarinic receptors and that nitric oxide and prostaglandins are mediators of this effect.

Our results are summarized below.

(A) Vascular endothelium is a necessary, and possibly the sole, component of intravascular acetylcholine dromotropic effects. This conclusion is based on the likelihood that the dextran–6AC–ABT complex, a muscarinic receptor blocker, remains intravascularly confined and that it is not hydrolyzed into free ABT during its passage through the coronary vasculature. Studies of diffusion of macromolecular dextrans of variable sizes across the vascular endothelium have generated a 'pore' model (Taylor and Granger, 1984). In these studies, intracoronary perfusions of macromolecular dextrans in dog heart have allowed the definition of an upper dimensional limit for capillary pore diameter of 0.024 to 0.032 μm (Taylor and Granger, 1984). The $2 \times 10^6$ Da dextran we used has an estimated molecular diameter $\geq 0.1$ μm; consequently, it could not permeate the capillary wall and the dextran-muscarinic blocker complex, being intravascular, could only act on luminal endothelial acetylcholine receptors (ach molecular diameter $\approx 0.54$ nm).

The control experiments showed that the dextran–6AC–ABT complex is responsible for the blockade of intravascular endothelial muscarinic receptors. We found no effect of dextran–6AC complex (control 1) and the possibility of non-carbodiimide adsorption of ABT was ruled out because control solution 2 was without acetylcholine blocking effect. Finally, venous effluents from hearts perfused with dextran–6AC–ABT, when treated with benzene to extract possible free ABT, showed that infusion of the benzene-extracted fraction (control 3) into an assay heart had no effect on acetylcholine-induced dromotropic effects, indicating that the effect of dextran–6AC–ABT is not the result of formed free ABT because, under our experimental conditions, the dextran–6AC and ABT bond is stable. In summary, our results showed that the competition between intravascular acetylcholine and the dextran–6AC–ABT complex shifts in a dose-dependent manner the acetylcholine A–V delay-curve downwards and to the right, indicating that there are endothelial muscarinic receptors in the blood vessel lumen.

(B) The finding that intracoronary injections of acetylcholine activates luminal endothelial muscarinic receptors indicates the existence of an indirect negative dromotropic effect via release of mediators with dromotropic effects.

It has been proposed that muscarinic receptor activation through changes in intracellular $CA^{2+}$ causes activation of a NO synthase, responsible for the synthesis of the potent vasodilator NO from L-arginine (Parkington et al., 1990). Synthesis of NO can be inhibited by L-arginine analogues such as $N^G$-nitro-L-arginine methyl ester (L-NAME) (Rees et al., 1989) and the accumulation of NO can be reduced by the NO scavenger, oxyhemoglobin (Martin et al., 1985). On the other hand, NO accumulation can be increased with NO-sparing agents such as superoxide dismutase which retards its degradation (Frangos and Juchan, 1991) or dithiothreitol which increases the NO half-life by acting as an antioxidant agent. Our results with these agents show that L-NAME and oxyhemoglobin reduced, while the NO-sparing agents potentiated the intravascular acetylcholine-induced dromotropic effects. These results indicate that intracoronary acetylcholine, acting on luminal endothelial receptors, stimulates synthesis and release of NO, which in turn may be responsible for slowing the impulse propaga-

tion in the A–V node since intracoronary infusion of NO or DEA–NO, a NO-donor, causes a negative dromotropic effect, these results do not exclude the existence of acetylcholine receptors and its direct effects in cardiac myocytes (Habuchi et al., 1996)

It is well known that endothelium-derived relaxing factor and NO induce transient hyperpolarization of vascular smooth muscle as a result of an increase in $K^+$ conductance (Beny, 1990) and an increase in $K^+$ outward current (Tare et al., 1990). It is possible that the same mechanisms may be involved in the transient A–V blockade induced by acetylcholine. In addition, in the A–V node as a result of direct application of acetylcholine on the AV node and isolated AV nodal cells there is a hyperpolarizing response and a decrease in slow $Ca^{2+}$ inward current (Hiromasa et al., 1983; Hancox et al., 1993; Han et al., 1996).

However, NO may not be the sole mediator of the dromotropic effect of intravascular acetylcholine because L-NAME did not totally block its effects, suggesting the existence of other mediators. Similarly, the results obtained by other investigators with isolated blood vessel preparations suggest the existence and participation of other endothelium-derived factor(s) in addition to NO because the hyperpolarizing endothelium-dependent acetylcholine response is not fully blocked by L-NAME and only partially inhibited by oxyhemoglobin (Komori et al., 1988; Parkington et al., 1990). Thus, we decided to turn to the possible role of the prostaglandin pathway as a source of additional endothelial mediators.

(C) Our experiments showed that inhibition of cyclooxygenase, a prostaglandin-forming enzyme, by indomethacin potentiated the acetylcholine effects and that this potentiation was totally reversed by L-NAME. These results contrast with those obtained when adenosine is used as a negative dromotropic agent. This agonist also acts via activation of intravascular-adenosine endothelial receptors (Balcells et al., 1992; Balcells and Rubio, 1993). In this case, indomethacin and L-NAME both depressed the effects of adenosine, and their effects were additive (unpublished results). These findings suggest that adenosine causes the release of a prostaglandin-like compound with negative dromotropic effects and that the adenosine-induced release of this compound and the release of NO proceed independently. The results with acetylcholine can be interpreted in several ways: (1) acetylcholine causes the release of a prostaglandin-like compound which is a positive dromotrope that superimposes its action on the negative effects of the enhanced NO release, i.e., both the NO and prostaglandin synthesis pathways are simultaneously and independently activated by acetylcholine. (2) Acetylcholine causes the release of a prostaglandin-like compound that in turn inhibits the activation of NO synthesis, i.e., both the NO and prostaglandin synthesis pathways are simultaneously activated by acetylcholine but are interindependent. Evidence indicates that NO and the arachidonic metabolic pathways may interact; for example, it has been shown that arachidonic acid metabolites, epoxyeicosatrienoic acids, enhance NO production by stimulating $Ca^{2+}$ influx into vascular endothelial cells (Graier et al., 1995). Nevertheless, it is clear that both acetylcholine and adenosine exert an endothelium-mediated negative inotropic effect that involves NO and prostaglandin synthesis (Nagoshi et al., 1998). However, if each of these agonist simply caused simultaneous receptor-mediated activation of the two pathways, their dromotropic effects should be equally affected by L-NAME and indomethacin. It is clear that further work is necessary to clarify these effects of intravascular acetylcholine, and since its dromotropic effects could not be completely blocked there must be still other important dromotropic mediator(s) of endothelial origin that need to be identified.

Our results disagree with those of Elvan et al. (1997) who showed a lack of effects of $N^G$-monomethyl-L-arginine (L-NMMA), a nitric oxide synthase inhibitor, on acetylcholine-induced dromotropic effects in dogs. The differences can be explained in several ways: (1) different duration of acetylcholine infusion (ours: 0.4 s; theirs: several seconds), (2) dose of acetylcholine used (ours: submaximal; theirs: supramaximal) or (3) species difference (guinea pig vs. dog).

In summary, our results demonstrate that intravascular acetylcholine, when acting on luminal endothelial muscarinic receptors causes a negative dromotropic effect mediated by NO, a possible prostanoid plus by other unknown mediators of endothelial origin.

## Acknowledgements

Supported by Grants from the Consejo Nacional de Ciencia Y Tecnologia No. 0433P-N and NIH RO1 HL47367-03.

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