Endocardial endothelium mediates luminal ACh-NO signaling in isolated frog heart

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Gattuso, Alfonsina, Rosa Mazza, Daniela Pellegrino, and Bruno Tota. Endocardial endothelium mediates luminal ACh-NO signaling in isolated frog heart. *Am. J. Physiol.* 276 (*Heart Circ. Physiol.* 45): H633–H641, 1999.—ACh exerted a biphasic effect in the in vitro working heart of *Rana esculenta*. High concentrations (10^{-7} M) of ACh depressed stroke volume (SV) and stroke work (SW) by ~30% with a shorter systolic phase and reduced peak pressure. Doses from 10^{-10} M induced a positive response peaking at 10^{-8} M (SV: +8.6%; SW: +6.5%) and a prolonged systolic phase without affecting peak pressure. Atropine and pirenzepine blocked both the positive and the negative effects of ACh. Pretreatment with Triton X-100 (0.1 ml, 0.05%) or with nitric oxide (NO)-cGMP pathway antagonists (N^G-nitro-L-arginine, N^G-nitro-L-arginine methyl ester, N^G-monomethyl-L-arginine, and 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one) abolished the positive and negative cholinergic effects. Infusion of 8-bromoguanosine 3′,5′-cyclic monophosphate reverted the positive effect of ACh to a negative effect. Milrinone blocked the positive inotropism but did not change the negative cholinergic response. The NO donor 3-morpholinosydnonimine generated a biphasic dose-response curve with a maximum positive effect at 10^{-8} M (SV: +8%; SW: +5.6%; systolic phase: +28 ms) and a negative effect at 5×10^{-8} M (SV and SW: about −12%; systolic phase: −70 ms; peak pressure: −1.50 mm). We conclude that in the avascular frog heart the endocardial endothelium mediates the inotropic effect of luminal cholinergic stimuli via a NO-cGMP pathway.

acetylcholine; nitric oxide; signal transduction

IN ADDITION to the well-known negative inotropism induced by ACh (26), a biphasic inotropic response to exogenous ACh has been sporadically reported in both amphibian and mammalian hearts (6, 24). The cellular and subcellular mechanisms governing this biphasic response are poorly understood. More recent studies showed that the ACh-stimulated isolated rabbit heart releases nitric oxide (NO) (2) and that myocytes themselves contain a constitutive NO synthase (30). Subsequently, studies with diverse kinds of mammalian cultured endothelial and myocardial cells suggested that ACh affects myocardial contractility via an NO signal transduction pathway. Thus NO appears to modulate intramyocardial cGMP levels and hence contractility (3, 19), thereby acting as a short-distance bidirectional messenger between endocardial endothelium (EE) cells and myocytes (5). However, because of a rapid loss of ACh receptors in some culture conditions, the results may not reflect in vivo conditions (7, 23). In the intact working heart the EE membrane is subjected to more distension and pressure than in cultured cell systems, and mechanical stresses induce the release of several factors that affect cardiac performance (31, 35). Thus studies of the response of the EE to ACh conducted in the whole working cardiac pump might provide more reliable information on the biphasic inotropic response sporadically found in amphibian and mammalian hearts.

In the coronary-supplied heart of homeotherms, not only does the coronary vascular endothelium constitute an almost contiguous stretch of tissue with the EE, but it seems to affect the contractile behavior of the adjacent myocardium in a manner similar to that of the EE so that the effects of both endothelial tissues appear additive and complementary (5, 29). Therefore, in whole heart studies of homeotherms it is difficult to dissect out an EE-mediated intracavitary autoregulation of cardiac performance via a receptor-mediated mechanism such as that involving an ACh-NO pathway.

The avascular heart of the frog, isolated and working at physiological loads, is an ideal system with which to explore the specific autocrine role of the EE as a source of NO without the confounding effects of the vascular endothelium (33). In fact, the frog EE is unique in being the only endothelial barrier between the superfusing blood and the myocardial microenvironment; only substances from the blood cross this barrier and interact with the myocardium (32).

The aim of this study was to analyze the role of the EE in the ACh-stimulated isolated and working frog heart. We demonstrate that in this heart preparation of *Rana esculenta*, an intact EE is necessary for the generation of the biphasic inotropic action of ACh and that this action involves an NO-cGMP signal transduction mechanism. Some preliminary results of this study have appeared in abstract form (11).

METHODS

Isolated and Perfused Working Heart Preparation

Frog hearts were isolated from specimens of both sexes of *R. esculenta* [weighing 22.015 ± 1.2 g (mean ± SE)] and connected to a perfusion apparatus as previously described (1, 33). Experiments were done at room temperature (18–21°C) from autumn to spring. A Grass S44 stimulator was used (single pulses of 20 V, 0.1 s) to stimulate electrically paced heart preparations; the stimulation rate was identical to the control (nonpaced) rate. The saline composition was (in mM) 115 NaCl, 2.5 KCl, 1.0 $CaCl_2$, 2.15 Na_2HPO_4, 0.85 NaH_2PO_4, and 5.6 anhydrous glucose; pH was adjusted to 7.20 by adding Na_2HPO_4 (10). The saline was equilibrated

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with air. Mean input pressure and minimal output pressure (diastolic afterload) were regulated by moving the reservoirs up or down with reference to the level of the atrium and the aortic trunk, respectively.

Measurements and Calculations

Pressure was measured through T tubes placed immediately before the input cannula and after the output cannula, using two MP-20D pressure transducers (Micron Instruments, Simi Valley, CA) connected to a Unirecord 7050 (Ugo Basile, Comerio, Italy). Pressure measurements were expressed in kilopascals and corrected for cannula resistance. Heart rate was calculated from pressure recording curves. Cardiac output was collected over 1 min and weighed; values were corrected for temperature and fluid density and expressed as volume measurements. The afterload (mean aortic pressure) was calculated as 2/3 diastolic pressure + 1/3 maximum pressure. Cardiac output and stroke volume (cardiac output/heart rate) were normalized per kilogram of wet body weight. Stroke volume at constant pre- and afterload in paced hearts was used as a measure of ventricular performance. Changes in stroke volume under these conditions were considered inotropic effects.

Ventricular stroke work, an index of systolic functionality, was calculated as (afterload − preload) × stroke volume/ventricle weight (mJ/g). The duration of the systolic phase and the height of peak pressure were calculated from recording traces.

Experimental Protocols

Basal conditions. In all experiments the diastolic afterload pressure was set at 3.92 kPa (40 cmH_2O), and the input pressure was regulated to obtain a cardiac output of ~110 $ml \cdot min^{-1} \cdot kg$ wet body wt^{-1}. These values are within the physiological range (33). The heart generated its own rhythm. Cardiac output, heart rate, and aortic pressure were measured simultaneously during the experiments. Hearts that did not stabilize within 10 min from the onset of perfusion were discarded. The basal condition parameters of cardiac performance were measured after a 20-min perfusion. These parameters are stable for >1 h (1, 33).

After the 20-min control period, the treated hearts were perfused for 20 min with drug (ACh or SIN-1)-enriched saline. Each heart was tested for one concentration of the drug. The ACh-stimulated hearts to be tested for other drugs were perfused a second time with the "medicated" Ringer, which contained ACh plus an inhibitor [atropine, pirenzepine, N^G-nitro-L-arginine methyl ester (L-NAME), N^G-nitro-L-arginine (L-NNA), N^G-monomethyl-L-arginine (L-NMMA), methylene blue, 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ), milrinone, diltiazem] or 8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP), after which the performance parameters were measured. These values were not significantly different when the second perfusion step lasted as much as 40 min.

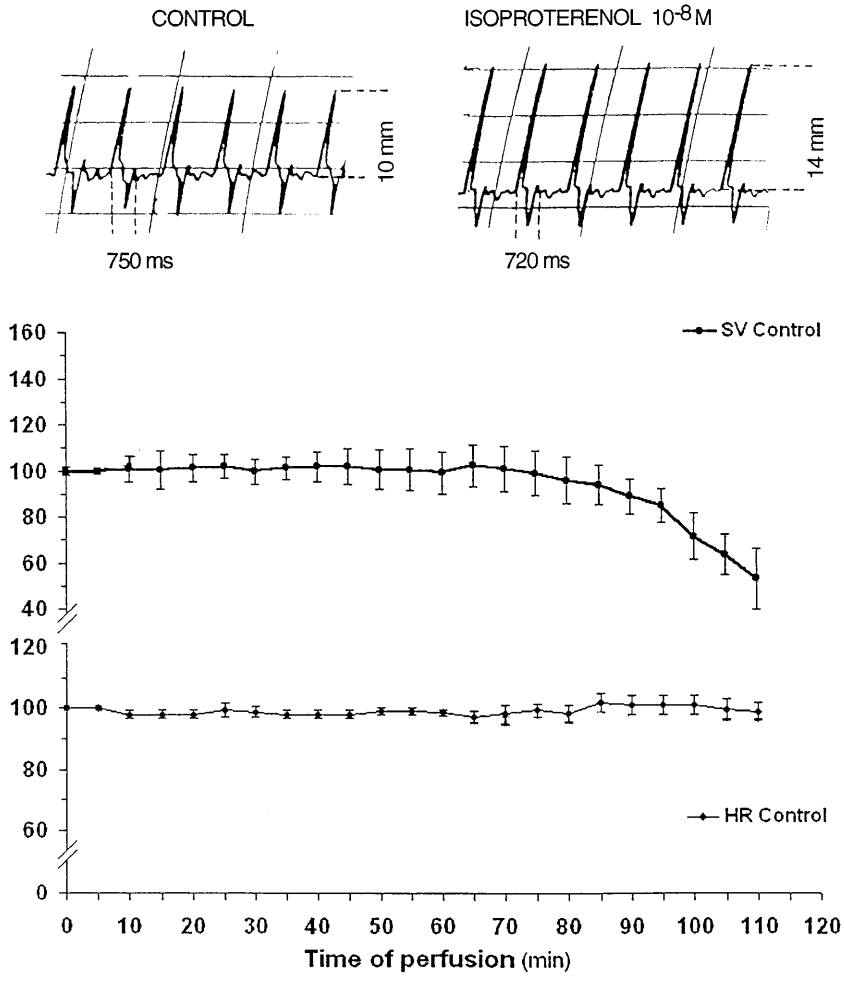

Fig. 1. *Top panels*: 2 typical pressure recording traces under control conditions (*left*) and after addition of isoproterenol (*right*). Dotted lines indicate systolic phase and peak pressure. *Bottom panel*: average time course of stroke volume (SV; ml/kg) and heart rate (HR; beats/min) in control conditions (n = 5 experiments). Before averaging (means ± SE), data were expressed as percentage of value at 5 min of perfusion, i.e., after stabilization. These baseline values were 2.3 ± 0.12 for SV and 51 ± 2.8 for HR.

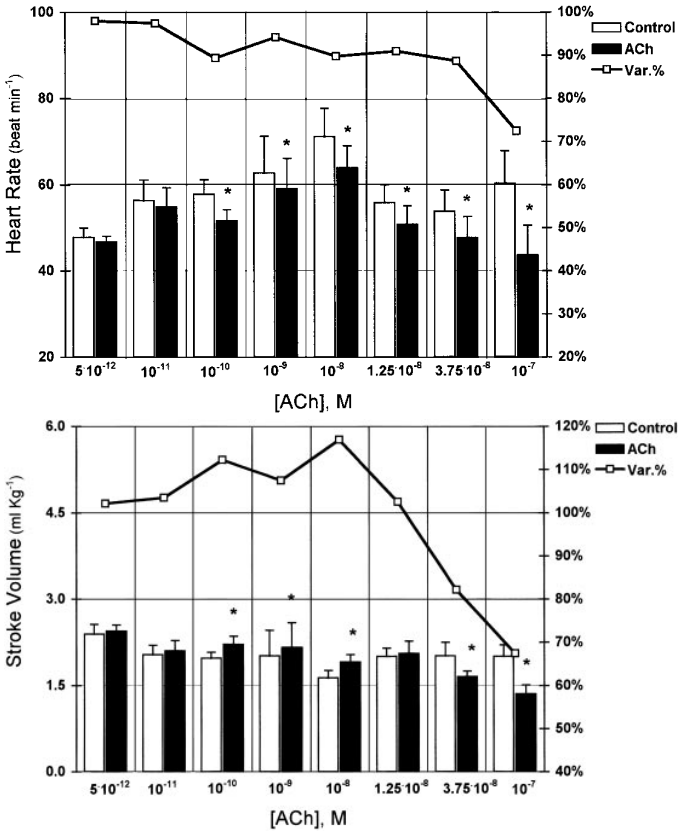

Fig. 2. Dose-response curve for ACh on HR and SV in isolated, perfused nonpaced frog hearts. Data are means ± SE of 3 [5×10^{-12} M ACh concentration ([ACh])], 4 (10^{-11} M), 4 (10^{-10} M), 3 (10^{-9} M), 6 (10^{-8} M), 3 (1.25×10^{-8} M), 4 (3.75×10^{-8} M), and 5 (10^{-7} M) experiments. Line graph, percent change; bars, actual mean values ± SE. Percent changes from control values from 5×10^{-12} to 10^{-7} M were −2.02 ± 1.01, −2.53 ± 0.89, −10.31 ± 3.7, −5.79 ± 0.51, −9.58 ± 3.45, −9.16 ± 1.04, −11.67 ± 2.98, and −28.44 ± 4 for HR and +2.1 ± 1.05, +3.42 ± 1.32, +12.56 ± 4.75, +8 ± 2.66, +16.17 ± 2.66, +2.36 ± 5.18, −17.1 ± 6.25, and −31 ± 7.5 for SV. *$P < 0.05$ vs. control value.

Functional impairment of EE. Ten to fifteen minutes after the onset of perfusion, when the heart was stabilized at basal conditions, 0.1 ml of Triton X-100 at a concentration of 0.05% was introduced through the aortic trunk, to avoid damage to the atrium, as follows. The inflow was closed, the afterload was simultaneously increased to ~7 kPa, and Triton X-100 was injected through a needle inserted in the output cannula so that the ventricle filled retrogradely because of a temporary incompetence of the valve. After three or four isovolumetric systoles, the inflow was opened and the outflow was adjusted to the control value, the perfusion being continued with the saline. Preliminary experiments with Evans blue dye showed that no backflow into the atrium occurs with this procedure. Parameters of cardiac performance were measured after 20 min of perfusion with the saline. In the hearts pretreated with detergent that were to be tested for the effects of ACh, this 20-min period was followed by a 20-min perfusion with the ACh-enriched saline, after which the performance parameters were measured.

Statistics

Results are expressed as means ± SE. Each heart received only one concentration of the drug being tested, under control conditions. Because each heart represented its own control, the statistical significance of differences was assessed on parameter changes using the paired Student's *t*-test ($P < 0.05$). Percent changes were evaluated as means ± SE of percent changes obtained from individual experiments.

Drugs and Chemicals

All the substances used were purchased from Sigma Chemical (St. Louis, MO). They were prepared as stock solutions in double-distilled water (ODQ was prepared in ethanol); dilutions were made in saline just before use. The following drugs were used: acetylcholine chloride, atropine sulfate salt, pirenzepine dihydrochloride, propanolol hydrochloride, phentolamine hydrochloride, Triton X-100, L-NAME, L-NNA, L-NMMA, methylene blue, ODQ, 8-BrcGMP sodium salt, milrinone [1,6-dihydro-2-methyl-6-oxo-(3,4′-bipyridine)-5-carbonitrile], 3-morpholinosydnonimine (SIN-1), diltiazem hydrochloride, and isoproterenol.

RESULTS

Isolated Working Heart Preparations

The functional characteristics of the preparation compare well with mechanical performance in frog heart (33). For example, the preparation had a clear positive inotropic response to the adrenergic agonist isoproterenol (10^{-8} M) in terms of both increased peak pressure and shorter systolic phase. Time-course experiments for stroke volume and heart rate in control conditions in nonpaced preparations have indicated that the performance of the frog heart was stable for at least 1 h (33) (Fig. 1).

ACh-Stimulated Preparations

The dose-response curve was generated by exposing each heart preparation to a single concentration of the drug, because exposure to ACh desensitizes myocyte preparations to subsequent ACh treatment (36).

Doses of ACh were from 5×10^{-12} to 10^{-7} M. At higher concentrations of ACh all hearts were irreversibly blocked. Doses of 10^{-10} M and higher caused a significant dose-dependent negative chronotropic effect (Fig. 2, *top*) paralleled by a remarkable decrease of cardiac output (data not shown). ACh exerted a biphasic effect on stroke volume, i.e., a gradual increase up to 10^{-8} M, followed by a dramatic decrease at immediately higher concentrations (Fig. 2, *bottom*). A dose-dependent curve was generated with electrically paced preparations to analyze this effect free from the chronotropic action of the substance. In preliminary control experiments, the hemodynamic parameters in the paced hearts were stable up to 1 h (data not shown). ACh clearly exerted a direct biphasic action on myocardial performance; a maximal positive effect on stroke volume and stroke work (Fig. 3) occurred at 10^{-8} M, followed by a sharp negative effect at 10^{-7} M. The effect on stroke volume and stroke work was stable and was readily reversed by perfusing with ACh-free Ringer solution. The duration of systolic phase was increased at lower concentrations (significant only at 10^{-8} M) and reduced at the higher dose (10^{-7} M) of ACh (Table 1).

Both the positive and the negative effect of ACh on stroke volume were mediated by muscarinic receptors.

This clearly emerges from experiments in which pretreatment with atropine (10^{-6} M) or pirenzepine (10^{-8} M) abolished the ACh-mediated changes of stroke volume (Fig. 4). Neither heart rate nor cardiac output was affected by atropine or pirenzepine alone (Table 2). It is noteworthy that the minimal concentrations of antagonists that abolished both these effects were very different, pirenzepine being more potent than atropine.

Negative inotropism by ACh and other muscarinic agonists is a well-recognized effect in the presence of cAMP-elevating agents. Pretreatment with α- and β-adrenergic antagonists (phentolamine and propanolol) blocked the positive and reduced the negative inotropic effect of ACh (Fig. 4). Interestingly, although pretreatment with the Ca^{2+} antagonist diltiazem abolished the negative effect of ACh (10^{-7} M), it reverted the positive effect of ACh (10^{-8} M) (Fig. 5). Diltiazem at a concentration of 10^{-8} M did not affect cardiac parameters, whereas at a higher concentration (10^{-7} M) it decreased both CO and HR.

Effects of Dysfunctional Ventricular Endocardial Endothelium

The Triton X-100 concentrations used in mammalian preparations (e.g., 0.5–1%) irreversibly blocked the frog heart; thus, we used lower concentrations. Transmission electron microscopy and confocal laser-scanning light microscopy showed that detergent treatment, as applied in this study, did not affect the morphology of the endothelial lining of the endocardium or of the subjacent myocardium (33).

After detergent pretreatment there was a significant reduction of heart rate and a mild increase of stroke volume, which persisted in the electrically paced hearts, whereas the endurance of the preparation remained unchanged for >1 h (33).

A dose-response curve of the effect of ACh on the detergent-treated hearts showed that both the positive and negative changes in stroke volume were abolished (Fig. 6). In contrast, the isoproterenol response in the detergent-treated hearts was unchanged (data not shown).

Involvement of NO Signaling System

The following series of experiments was designed to test whether the effects of ACh on the myocardial performance of the frog heart were mediated by an NO pathway. To determine whether the response to ACh was modified by inhibitors of NO synthase (NOS), ACh was perfused after hearts had been exposed to L-NAME (10^{-4} M), L-NNA (10^{-5} M) or L-NMMA (10^{-4} M). These NOS inhibitors blocked the cholinergic effects on both stroke volume and stroke work (Fig. 7). We then tried to obtain information on the putative involvement of guanylate cyclase in this process. Although the nonspecific inhibitor methylene blue (10^{-6} M) and the specific inhibitor ODQ (10^{-5} M) abolished both the positive and the negative effects of ACh on stroke volume, the stable and diffusible analog of cGMP, 8-BrcGMP (10^{-6} M), shifted the negative myocardial effect of ACh to lower doses (Fig. 7). The infusion with milrinone (10^{-5} M), which specifically blocks the cGMP-inhibited cAMP phosphodiesterase (cG_i-PDE or PDE3), abolished the positive effect of ACh but not the negative one (Fig. 8). Perfusion with the NO donor SIN-1 mimicked the biphasic dose response of ACh (Fig. 9 and Table 1).

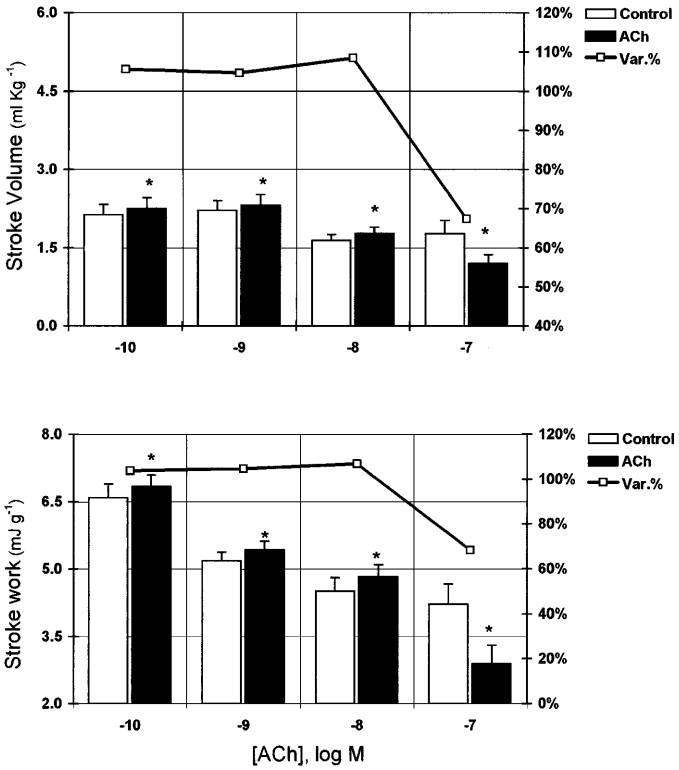

Fig. 3. Dose-response curve for ACh on SV and stroke work in isolated and perfused paced frog hearts. Data are means ± SE of 3 (10^{-10} M [ACh]), 4 (10^{-9} M), 6 (10^{-8} M), and 5 (10^{-7} M) experiments. Line graph, percent change; bars, actual mean values ± SE. Percent changes from control values from 10^{-10} to 10^{-7} M were +5.32 ± 0.13, +4.65 ± 1.27, +8.6 ± 1.4, and −31.7 ± 3.79 for SV and +3.6 ± 0.92, +3.56 ± 0.86, +6.58 ± 1.71, and −33 ± 4 for stroke work. *$P < 0.05$ vs. control value.

DISCUSSION

The response to hemodynamic stimuli of the in vitro heart preparation used in this study mimics the response of the in vivo heart. When the isolated, perfused

Table 1. *Duration of systolic phase and height of peak pressure in control conditions and after treatment with Iso, ACh, and SIN-1*

Intervention	*n*	Δ Duration of Systolic Phase, ms	Δ Height of Peak Pressure, mm
Iso (10^{-8} M)	6	−25 ± 5‡	+3.50 ± 0.50†
ACh (10^{-8} M)	6	+45 ± 12†	−0.10 ± 0.50 NS
ACh (10^{-7} M)	5	−50 ± 10†	−0.44 ± 0.15*
SIN-1 (10^{-8} M)	4	+28 ± 11*	−0.37 ± 0.40 NS
SIN-1 (5×10^{-8} M)	5	−70 ± 25*	−1.50 ± 0.40*

Values are means ± SE; *n*, no. of hearts. Averaged control values of duration of systolic phase and height of peak pressure were 829 ± 30 ms and 6.83 ± 0.5 mm, respectively. Iso, isoproterenol; SIN-1, 3-morpholinosydnonimine; NS, not significant. Significant difference from control: *$P < 0.05$; †$P < 0.025$; ‡$P < 0.005$.

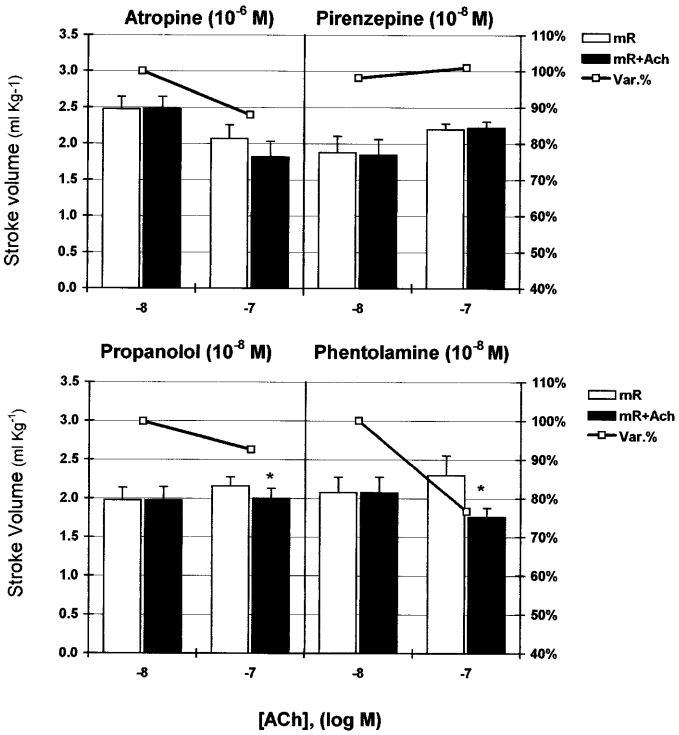

Fig. 4. Effects of ACh (10^{-8} and 10^{-7} M) after pretreatment with atropine (10^{-6} M) and pirenzepine (10^{-8} M) and after pretreatment with propanolol (10^{-8} M) and phentolamine (10^{-8} M) on SV in isolated and perfused paced frog hearts. Data are means ± SE of 4 experiments in each group. Line graphs, percent change; bars, actual mean values ± SE. Although treatment with muscarinic antagonists blocked both positive and negative cholinergic inotropic effect, treatment with adrenergic antagonists (propanolol and phentolamine) abolished positive inotropic effects and reduced negative inotropism of ACh from −31.7 ± 8.79 to −7.54 ± 1.66 for propanolol and to −22 ± 7.29 for phentolamine. MR, medicated Ringer. Neither atropine nor pirenzepine alone had any effect on cardiac parameters. Both propanolol and phentolamine alone significantly decreased SV (−9.4 ± 3.4 and −5.3 ± 1.7%, respectively) and SW (−10.0 ± 3.4 and −6.1 ± 2.6%, respectively). *$P < 0.05$ vs. control value.

working heart preparation of *R. esculenta*, set up and standardized by us (1, 33), was perfused at constant pressure, it generated physiologically comparable values of output pressure, cardiac output, ventricle work, and power and showed the typical "hypodynamic state" after a relatively constant time from the onset of the perfusion. With this preparation, we demonstrate that exogenous ACh induces a biphasic inotropic response consequent to the muscarinic receptor-mediated stimulation of the EE. Exposure of each cardiac preparation to a single concentration of ACh only avoids the confounding effects of desensitization (36).

The parasympathetic innervation of the frog heart is well documented; the ventricle exhibits a higher cholinergic nerve density than mammalian hearts (20), and the cholinergic varicosities are densely distributed on the surface of the myocytes, without synaptic specialization, and in the space between them (13), whereas the muscarinic receptors are randomly distributed over the entire cellular surface of the myocytes and also of the nonmyocyte components (13). These features suggested

Table 2. *Heart rate and cardiac output in control conditions and after treatment with atropine and pirenzepine*

	HR, beats/min	CO, ml·min^{-1}·kg^{-1}
Control	53.273 ± 4.235	114.580 ± 2.400
Atropine (10^{-6} M)	52.790 ± 3.875	114.032 ± 2.744
Control	59.953 ± 6.708	111.367 ± 1.997
Pirenzepine (10^{-8} M)	59.793 ± 6.463	113.600 ± 4.214

Values are means ± SE; $n = 4$ hearts each for atropine and pirenzepine studies. HR, heart rate; CO, cardiac output.

that ACh "bathes" the muscle, so that some aspects of the vagal effects on the whole working heart are obtained by perfusing heart preparations with ACh, rather than by "focal" (e.g., iontophoretic) application (20).

In addition to its classic negative inotropism, exogenous ACh, at concentrations of 10^{-5} M or higher, exerts a positive inotropic effect distinct from the well-known catecholamine-dependent positive inotropism of exogenous ACh (20) in mammalian (14, 6), avian (4), and amphibian (24) heart ventricle preparations. The mechanism of this positive inotropism was poorly understood until the recognition that stimulation of low-affinity myocardial muscarinic receptors produced a positive inotropic effect parallel to a rise in intracellular Na^+ activity (17) and leading to an increase in free intracellular Ca^{2+} concentration (18). Present evidence obtained in myocardial preparations indicates that these cholinergic-mediated inotropic effects result from the activation by ACh of cardiac muscarinic receptors coupled with G proteins, which in turn modifies the activity of second messenger pathways, Ca^{2+} homeostasis, ionic channels, and contractile proteins (8).

The putative role of EE cells in mediating such cholinergic stimuli in the intact heart has been ignored. Whereas in isolated myocytes the concentrations of ACh or carbachol were higher than those associated

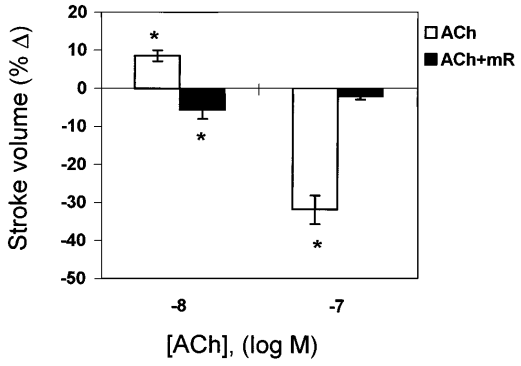

Fig. 5. Effects of ACh (10^{-8} and 10^{-7} M) after pretreatment with diltiazem (10^{-8} M) on SV in isolated and perfused paced frog hearts. Results are expressed as percent changes (%Δ) from control value. Data are means ± SE of 4 experiments in each group. Treatment with calcium antagonist induced a negative inotropism at a low concentration (10^{-8} M) (−6.52 ± 2.24) and abolished negative inotropic effect at a high concentration (10^{-7} M). Diltiazem (10^{-8} M) alone had no effect on cardiac parameters. *$P < 0.05$ vs. control value.

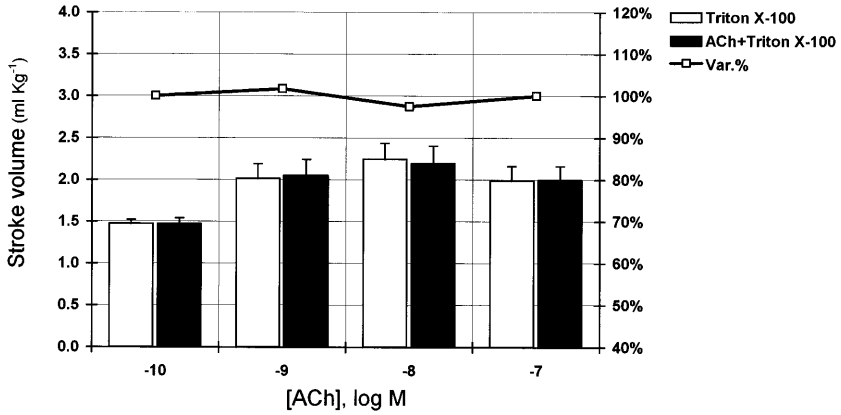

Fig. 6. Dose-response curve for ACh on SV after pretreatment with Triton X-100 (0.05%) in isolated and perfused paced frog hearts. Data are means ± SE of 3 experiments in each group. Line graph, percent change; bars, actual mean values ± SE. There were no significant changes (paired Student's *t*-test: $P = 1.0$).

with the negative inotropism of ACh, thereby supporting the conclusion that muscarinic agonists do not increase the force of contraction under physiological conditions (16), the opposite is true in our whole cardiac preparation. This illustrates how the effects of the various mechanisms responsible for the inotropic action of ACh can vary according to the experimental design. For example, whereas only mRNA for the M_2 receptor has been detected in cultured endothelial cells, mRNA for M_1-, M_2-, and M_3-receptor subtypes have been identified in freshly isolated endothelial cells (34). A critical reevaluation of previous studies using multicellular or isolated cardiomyocytes, in which the EE might have been mechanically impaired, is clearly needed.

Because of the relatively long exposure of the perfused cardiac preparation to ACh under our experimental conditions, the drug might act via various second-messenger signaling systems linked to the different muscarinic receptor subtypes, which, in addition to the predominant myocardial subtype M_2, include those of the EE, the first barrier involved in luminal stimulus-secretion coupling. Although atropine blockade cannot distinguish among muscarinic receptor subtypes, low concentrations of pirenzepine, an "M_1-selective" muscarinic ACh receptor (mAChR) antagonist, block only M_1 sites (15). Therefore, the finding that pirenzepine completely inhibited both the positive and the negative effects of ACh at concentrations two orders of magnitude lower than those of atropine indicates that M_1-

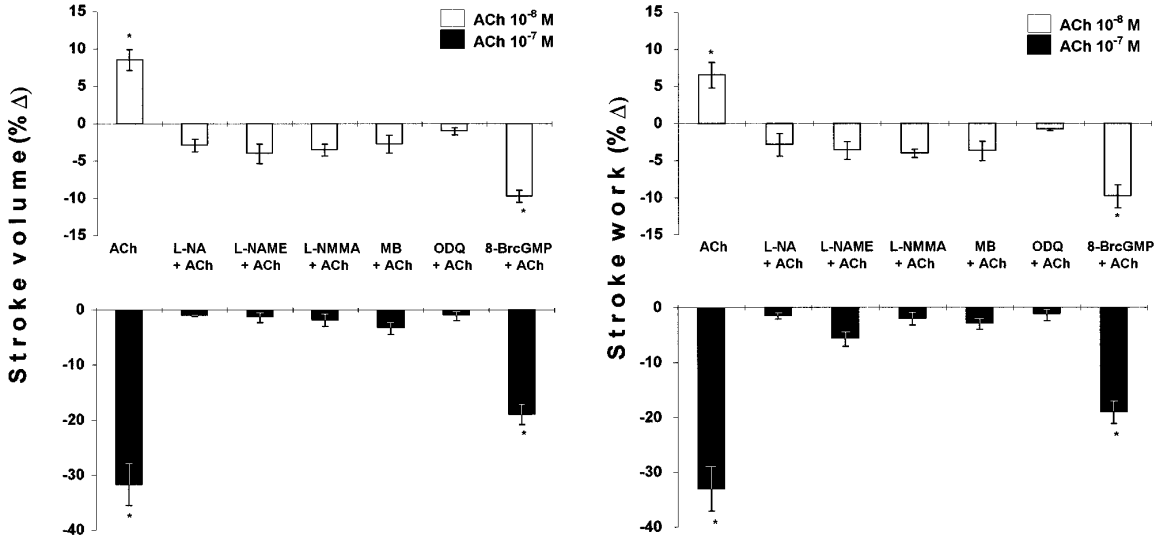

Fig. 7. Effects of ACh (10^{-8} and 10^{-7} M) after pretreatment with N^G-nitro-L-arginine (L-NNA, 10^{-5} M), N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M), N^G-monomethyl-L-arginine (L-NMMA, 10^{-4} M), methylene blue (MB, 10^{-6} M), 1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one (ODQ, 10^{-5} M), and 8-bromoguanosine 3′,5′-cyclic monophosphate (8-BrcGMP, 10^{-6} M) on SV and stroke work in isolated, perfused paced frog hearts. Results are expressed as percent change from control value. Data are means ± SE of 3–4 experiments in each group. Although treatment with L-NNA, L-NAME, L-NMMA, MB, and ODQ abolished cholinergic effects on both SV and stroke work, treatment with 8-BrcGMP induced a negative response at a low concentration (10^{-8} M) (−9.7 ± 0.8% for SV and −9.79 ± 1.5% for stroke work) and did not modify negative effects at a high dose (10^{-7} M). L-NNA, L-NAME, L-NMMA, MB, and ODQ, individually tested, induced significant increases of both SV (+3.84 ± 1.35, + 3.18 ± 1.02, +6.47 ± 1.58, +6.44 ± 2.11, and +5.74 ± 1.91%, respectively) and SW (+3.84 ± 1.35, +3.3 ± 1.06, +6.78 ± 2.19, +6.86 ± 2.55, and +5.15 ± 2.18%, respectively). 8-BrcGMP alone decreased both SV (−6.55 ± 2.94%) and SW (−7.21 ± 2.78%). *$P < 0.05$ vs. control value.

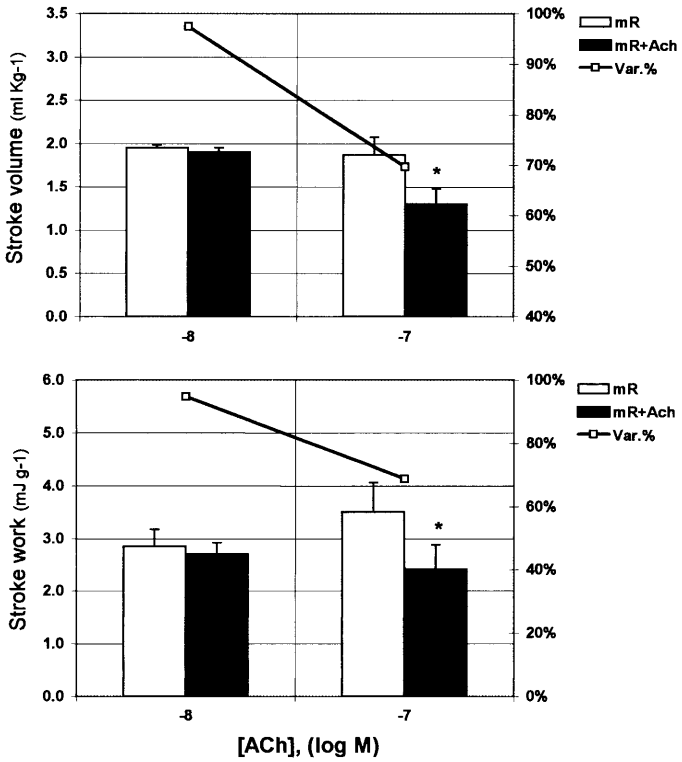

Fig. 8. Effects of ACh (10^{-8} and 10^{-7} M) after pretreatment with milrinone (10^{-5} M) on SV and stroke work in isolated, perfused paced frog hearts. Data are means ± SE of 4 experiments in each group. Line graph, percent change; bars, actual means values ± SE. Treatment with specific inhibitor of cG_i-PDE abolished positive inotropic effect without affecting negative effect. *$P < 0.05$ vs. control value.

type muscarinic receptors play an important role in the transduction of cholinergic signals in the EE of the frog heart ventricle.

In the isolated ventricular myocytes of *R. esculenta*, atropine and other "M_2-selective" antagonists exert an intrinsic negative effect on mAChR, i.e., they reduce the interaction between the receptor and the G (G_i and G_k) proteins, and more importantly, they stimulate the L-type calcium current previously stimulated by isoprenaline and inhibit the mAChR-activated K^+ current (12). Consequently, we tested the effects on our system of different periods of perfusion of atropine or of pirenzepine. As shown in Table 2, neither of these antagonists affected the basal hemodynamic parameters of the preparations.

ACh, via M_2 muscarinic receptors coupled to G proteins, directly activates $I_{K,ACh}$ (the pathway probably responsible for the basal increase in K^+ current elicited by ACh) (21). However, in a variety of cell tissues, ACh also acts via M_1- and M_3-receptor subtypes coupled to G proteins to stimulate phospholipase C and the hydrolysis of phosphatidylinositol (PI) (28). The hydrolysis of PI elicits the production of second messengers, which may modulate channel function. Very recently, molecular cloning and ectopic expression of muscarinic receptor subtypes have provided further evidence that although M_2 and M_4 subtypes are principally coupled to adenylate cyclase inhibition, M_1-, M_3-, and M_5-receptor subtypes are functionally coupled to mobilization of intracellular Ca^{2+} (8, 15). It is thus of interest that the positive inotropism of ACh is reversed into negative inotropism when the frog heart is pretreated with the calcium antagonist diltiazem.

In studies on intact ventricular preparations, the negative inotropic effects of muscarinic agonists were most evident under conditions of elevated cAMP (3, 22, 26, 27). The experiments with α- and β-adrenergic antagonists revealed that adrenergic (intrinsic) tone is involved in the effects exerted by ACh. In fact, pretreatment with the β-blocker propanolol and the α-blocker phentolamine abolished the positive and reduced the negative cholinergic inotropism.

Both cholinergic antagonist pretreatment and functional impairment of the ventricular EE by Triton X-100 abolished the biphasic inotropic response of ACh. Under our experimental conditions, 0.1 ml of 0.05% Triton X-100 was injected in the aortic trunk to perfuse the ventricle, but not the atrium, through the temporarily incompetent valve. This concentration does not cause structural or ultrastructural changes in EE cells (33). It is noteworthy that when only the ventricular luminal surface was treated with detergent, pacemaker activity and atrial myocardial mechanical and endothelial secretory performance remained intact (33). This mild treatment with Triton X-100 blocked cholinergic stimulation as effectively as did antagonist pretreatment, a result that illustrates the high sensitivity of

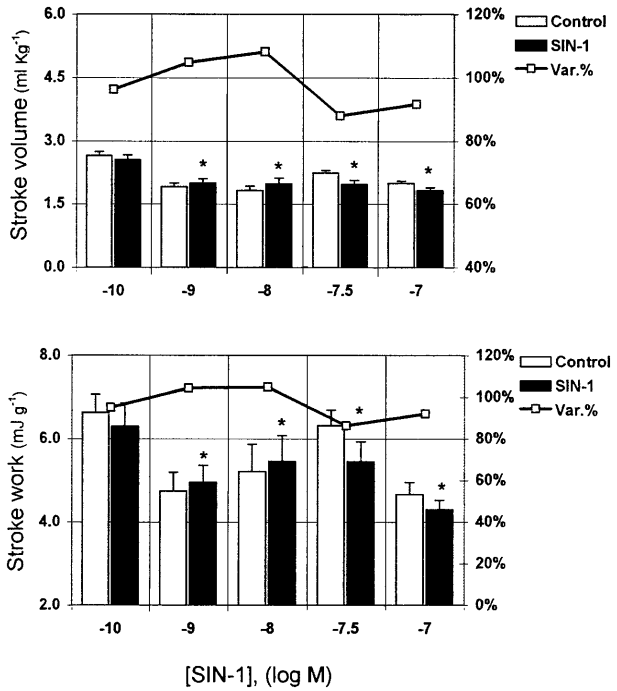

Fig. 9. Dose-response curve for 3-morpholinosydnonimine (SIN-1) on SV and stroke work in isolated, perfused nonpaced frog hearts. Data are means ± SE of 3 [10^{-10} M SIN-1 concentration ([SIN-1])], 4 (10^{-9} M), 4 (10^{-8} M), 5 (5×10^{-8} M), and 5 (10^{-7} M) experiments. Line graph, percent change; bars, actual mean values ± SE. Percent changes from control values from 10^{-10} to 10^{-7} M were −4.09 ± 1.7, +4.97 ± 1.15, +8.17 ± 1.91, −12.17 ± 3.77, and −7.83 ± 3.21 for SV and −5.1 ± 2.21, +4.97 ± 1.66, +5.63 ± 2.34, −13.28 ± 3.33, and −7.45 ± 2.76 for stroke work. *$P < 0.05$ vs. control value.

muscarinic receptors to their microenvironment, which is also well documented by the loss of antagonist selectivity after detergent solubilization (15). Therefore, the functional integrity of the EE appears to be a prerequisite for the transduction of blood-borne cholinergic signals to the myocardium.

We also demonstrate that cholinergic-mediated stimulation of the EE involves an NO signaling system by which EE cells are triggered to release NO or a nitroso compound that in turn might affect guanylate cyclase activity. In fact, pretreatment with either specific NOS inhibitors (L-NAME, L-NNA, and L-NMMA) or aspecific (methylene blue) or specific (ODQ) inhibitors of guanylate cyclase abolishes the biphasic inotropic action of ACh, as does cholinergic blockade.

There is controversy as to the inotropic effects of NO donors. Negative inotropism of sodium nitroprusside has been observed in some cardiac preparations but not by others (22). To our knowledge, ours is the only study to show the biphasic inotropism of SIN-1 on an intact cardiac preparation. The finding that SIN-1 exerts an inotropic biphasic dose-response curve identical with that induced by ACh coincides with the hypothesis that both these cGMP-elevating agents (22, 27) act via an NO-dependent mechanism.

Taken together, these results indicate that the EE of the frog heart ventricle is an important cellular source of the NO signal under basal conditions (33), but when stimulated it exerts a paracrine effect on the subjacent myocytes, possibly through a modulation of intracellular messengers such as cGMP. This possibility is supported by the finding that with 8-BrcGMP preincubation the negative inotropic response occurred at lower doses of ACh (i.e., 10^{-8} M).

Such functional NO-mediated responses could result from the integrated activation of a variety of signal transduction pathways, involving, for example, a rise in intracellular calcium, stimulation of guanylate cyclase, and increase of cGMP levels, with in turn feedback modulation of intracellular calcium and cAMP levels via the cGMP-dependent cAMP phosphodiesterases. In isolated rat ventricular myocytes, both muscarinic cholinergic and β-adrenergic stimulations are mediated, at least in part, by NO (3). In their study on the isolated ventricular myocytes of the frog (*R. esculenta*), Méry et al. (27) demonstrated a biphasic transsarcolemmal calcium current (I_{Ca}) response to NO donors, which was excitatory or inhibitory depending on the nanomolar or micromolar ranges of concentration of the NO donor, respectively. Both of these stimulatory and inhibitory effects appeared to be mediated by NO and by the consequent accumulation of cGMP not only via the "soluble" NO-sensitive guanylyl cyclase but possibly via the membrane-bound isoform of the enzyme. In the light of these results and previous data on a cG_i-PDE or PDE3 (cGMP-inhibited cAMP phosphodiesterase) functionally coupled to the L-type calcium channel in the frog heart (9, 25), Méry et al. (27) suggested that guanylyl cyclase and cGMP can play a role in the fine tuning of cardiac cAMP concentration by positive and negative controls via inhibition of the cG_i-PDE and stimulation of the cG_s-PDE, respectively. In agreement with the aforementioned data, our finding that milrinone, a specific inhibitor of PDE3, blocks the positive inotropism of ACh without affecting the negative one suggests a role for cG_i-PDE in our system.

In conclusion, our results document in an isolated working frog heart preparation that a functionally intact EE is necessary to activate the signal transduction pathway interposed between the blood-borne cholinergic stimuli acting on the muscarinic receptors of the luminal side of the endothelial cells and the contractile machinery of the subjacent ventricular myocytes. In fact, the selective damage of the ventricular EE with Triton X-100 completely abolished the biphasic cholinergic response in the same way that atropine and pirenzepine block muscarinic receptors. This pathway includes an NO signaling system present in the EE cells that is involved in both the positive and negative inotropic responses to ACh.

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REFERENCES

1. **Acierno, R., A. Gattuso, M. C. Cerra, D. Pellegrino, C. Agnisola, and B. Tota.** The isolated and perfused working heart of the frog, *Rana esculenta*: an improved preparation. *Gen. Pharmacol.* 25: 521–526, 1994.
2. **Amezcua, J. L., G. J. Dusting, R. M. J. Palmer, and S. Moncada.** Acetylcholine induces vasodilation in the rabbit isolated heart through the release of nitric oxide, the endogenous nitrovasodilator. *Br. J. Pharmacol.* 95: 830–834, 1988.
3. **Balligand, J. L., R. A. Kelly, P. A. Marsden, and T. W. Smith.** Control of cardiac muscle cell function by an endogenous nitric oxide signaling system. *Proc. Natl. Acad. Sci. USA* 90: 347–351, 1993.
4. **Biegon, R. L., P. M. Epstein, and A. J. Pappano.** Muscarinic antagonism of the effects of phosphodiesterase inhibitor (methylisobutylxanthine) in embryonic chick ventricle. *J. Pharmacol. Exp. Ther.* 215: 348–356, 1980.
5. **Brutsaert, D. L., and L. J. Andries.** The endocardial endothelium. *Am. J. Physiol.* 263 (*Heart Circ. Physiol.* 32): H985–H1002, 1992.
6. **Buccino, R. A., E. H. Sonnenblick, T. Cooper, and E. Braunwald.** Direct positive inotropic effect of acetylcholine on myocardium. Evidence for multiple cholinergic receptors in the heart. *Circ. Res.* 19: 1097–1108, 1966.
7. **Busse, R., H. Fichtner, A. Luckhoff, and M. Kohlard.** Hyperpolarization and increased free calcium in acetylcholine-stimulated endothelial cells. *Am. J. Physiol.* 255 (*Heart Circ. Physiol.* 24): H965–H969, 1988.
8. **Felder, C. C.** Muscarinic acetylcholine receptors: signal transduction through multiple effectors. *FASEB J.* 9: 619–625, 1995.
9. **Fischmeister, R., and H. C. Hartzell.** Regulation of calcium current by low-K_m cyclic AMP phosphodiesterases in cardiac cells. *Mol. Pharmacol.* 38: 426–433, 1990.
10. **Flitney, F. W., and J. Singh.** Release of prostaglandins from the isolated frog ventricle and associated changes in endogenous cyclic nucleotide levels. *J. Physiol. (Lond.)* 304: 1–20, 1980.
11. **Gattuso, A., D. Pellegrino, R. Mazza, and B. Tota.** Inotropic effects of acetylcholine on frog heart with intact and damaged endocardium endothelium (Abstract). *J. Physiol. (Lond.)* 483: 185P, 1995.

12. **Hanf, R., Y. Li, G. Szabo, and R. Fischmeister.** Agonist-independent effects of muscarinic antagonists on Ca^{2+} and K^+ currents in frog and rat cardiac cells. *J. Physiol. (Lond.)* 461: 743–765, 1993.
13. **Hartzell, H. C.** Distribution of muscarinic acetylcholine receptors and presynaptic nerve terminals in amphibian heart. *J. Cell Biol*. 86: 6–20, 1980.
14. **Hollenberg, M., S. Carriere, and C. Barger.** Biphasic action of acetylcholine on ventricular myocardium. *Circ. Res*. 14: 527–536, 1965.
15. **Hulme, E. C., N. J. M. Birdsall, and N. J. Buckley.** Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol*. 30: 633–673, 1990.
16. **Korth, M., and V. Kuhlkamp.** Muscarinic receptor-mediated increase of intracellular Na^+ ion activity and force of contraction. *Pflügers Arch*. 403: 266–272, 1985.
17. **Korth, M., and V. Kuhlkamp.** Muscarinic receptors mediate negative and positive inotropic effects in mammalian ventricular myocardium: differentiation by agonists. *Br. J. Pharmacol*. 90: 81–90, 1987.
18. **Korth, M., V. K. Sharma, and S. S. Sheu.** Stimulation of muscarinic receptors raises free intracellular Ca^{2+} concentration in rat ventricular myocytes. *Circ. Res*. 62: 1080–1087, 1988.
19. **Lamontagne, D., U. Pohl, and R. Busse.** N^G-nitro-L-arginine antagonizes endothelium-dependent dilator responses by inhibiting endothelium-derived relaxing factor release in the isolated rabbit heart. *Pflügers Arch*. 418: 266–270, 1991.
20. **Loffeholz, K., and A. Pappano.** The parasympathetic neuroeffector junction of the heart. *Pharmacol. Rev*. 37: 1–24, 1985.
21. **Logothetis, D. F., Y. Kurachi, J. Galper, E. J. Neer, and D. E. Clapham.** The βγ subunits of GTP-binding proteins activate the muscarinic K^+ channel in the heart. *Nature* 325: 321–326, 1987.
22. **MacDonnell, K., G. F. Tibbits, and J. Diamond.** cGMP elevation does not mediate muscarinic agonist-induced negative inotropy in rat ventricular cardiomyocytes. *Am. J. Physiol*. 269 (*Heart Circ. Physiol*. 38): H1905–H1912, 1995.
23. **Manabe, K., H. Ito, H. Matsuda, and A. Noma.** Hyperpolarization induced by vasoactive substances in intact guinea-pig endocardial endothelial cells. *J. Physiol. (Lond.)* 484: 25–40, 1995.
24. **McDowall, R. J. S.** The stimulating action of acetylcholine on the heart. *J. Physiol. (Lond.)* 104: 392–403, 1946.
25. **Méry, P. F., V. Brechler, C. Pavoine, F. Pecker, and R. Fischmeister.** Glucagon stimulates the cardiac Ca^{2+} current by activation of adenylyl cyclase and inhibition of phosphodiesterase. *Nature* 345: 158–161, 1990.
26. **Méry, P.-F., L. Hove-Madsen, J.-M. Chesnais, H. C. Hartzell, and R. Fischmeister.** Nitric oxide synthase does not participate in negative inotropic effect of acetylcholine in frog heart. *Am. J. Physiol*. 270 (*Heart Circ. Physiol*. 39): H1178–H1188, 1996.
27. **Méry, P. F., C. Pavoine, L. Belhassen, F. Pecker, and R. Fischmeister.** Nitric oxide regulates cardiac Ca^{2+} current. Involvement of cGMP-inhibited and cGMP-stimulated phosphodiesterases through guanylyl cyclase activation. *J. Biol. Chem*. 268: 26286–26295, 1993.
28. **Nathanson, N. M.** Molecular properties of the muscarinic acetylcholine receptor. *Annu. Rev. Neurosci*. 10: 195–236, 1987.
29. **Ramaciotti, C., A. Sharkey, G. McClellan, and S. Winegrad.** Endothelial cells regulate cardiac contractility. *Proc. Natl. Acad. Sci. USA* 89: 4033–4036, 1992.
30. **Schulz, R., E. Nava, and S. Moncada.** Induction and biological relevance of a Ca^{2+}-independent nitric oxide synthase in the myocardium. *Br. J. Pharmacol*. 105: 575–580, 1992.
31. **Singh, J.** Stretch stimulates cyclic nucleotide metabolism in the isolated frog ventricle. *Pflügers Arch*. 395: 162–164, 1982.
32. **Stehbens, W. E., and E. Meyer.** Ultrastructure of endothelium of the frog heart. *J. Anat*. 99: 127–134, 1965.
33. **Sys, S. U., D. Pellegrino, R. Mazza, A. Gattuso, L. J. Andries, and B. Tota.** Endocardial endothelium in the avascular heart of the frog: morphology and role of nitric oxide. *J. Exp. Biol*. 200: 3109–3118, 1997.
34. **Tracey, W. R., and M. J. Peach.** Differential muscarinic receptor mRNA expression by freshly isolated and cultured bovine endothelial cells. *Circ. Res*. 70: 234–240, 1992.
35. **Tung, L., and S. Zou.** Influence of stretch on excitation threshold of single frog ventricular cells. *Exp. Physiol*. 80: 221–235, 1995.
36. **Wang, Y. G., and S. L. Lipsius.** Acetylcholine potentiates acetylcholine-induced increases in K^+ current in cat atrial myocytes. *Am. J. Physiol*. 268 (*Heart Circ. Physiol*. 37): H1313–H1321, 1995.