Positive Inotropic, Negative Chronotropic, and Coronary Vasoconstrictor Effects of Acetylcholine in Isolated Rat Hearts: Role of Muscarinic Receptors, Prostaglandins, Protein Kinase C, Influx of Extracellular Ca^{2+} , Intracellular Ca^{2+} Release, and Endothelium

Sadiye ATEŞ and Ziya KAYGISIZ

Department of Physiology, Faculty of Medicine, University of Osmangazi, 26480 Eskişehir, Turkey

Abstract: The involvement of nitric oxide (NO), muscarinic receptors, prostaglandins, calcium influx via slow calcium channels, Ca^{2+} release from intracellular stores, protein kinase C, and endothelium in the positive inotropic, negative chronotropic, and coronary vasoconstrictor effects of acetylcholine (ACh) has been investigated in isolated rat hearts. The perfusion of hearts with ACh (10^{-7} , 5×10^{-7} , and 10^{-6} M) produced marked decreases in heart rate and coronary flow and a marked increase in contractile force. Similar effects have been observed during the perfusion of hearts with ACh in the presence of N^ω-nitro-L-arginine methyl ester (L-NAME), which is an inhibitor of NO synthesis. The positive inotropic, negative chronotropic, and coronary vasoconstrictor effects of ACh were abolished by muscarinic receptor blocker atropine. In hearts pretreated with cyclooxygenase inhibitor indomethacin, ACh significantly decreased heart rate but did not significantly affect coronary flow and contractile force. In the presence of calcium channel antagonist verapamil or protein kinase C inhibitor staurosporine, ACh produced a significant drop in heart rate but did not significantly af-

fect coronary perfusion pressure and force of contraction. In the presence of the inhibitor of the release of Ca^{2+} from intracellular stores dantrolene sodium, ACh produced a significant increase in coronary perfusion pressure and a marked decline in heart rate, but did not significantly affect force of contraction. Furthermore, the disruption of endothelium by perfusing the hearts with saponin abolished the vasoconstrictor effect of ACh but did not alter negative chronotropic and positive inotropic effect. Our results suggest that ACh causes vasoconstrictor, negative chronotropic, and positive inotropic effects in isolated rat hearts. Cardiac effects of ACh are related to muscarinic receptor activation, and prostaglandins modulate ACh-induced vasoconstriction and positive inotropy. Our data also suggest that protein kinase C and calcium influx from extracellular source may be responsible for the vasoconstrictor and positive inotropic effect of ACh. The calcium release from intracellular stores may mediate the positive inotropic effect, and the vasoconstrictor effect of ACh depends on an intact endothelium. [Japanese Journal of Physiology, 48, 483–491, 1998]

Key words: acetylcholine, isolated perfused rat heart, myocardial contractility, heart rate, coronary flow.

It has been originally demonstrated by Furchgott and Zawadzki [1] that ACh produces endothelium-dependent relaxation of vascular smooth muscle. It has been

shown that ACh causes vasodilation in large blood vessels and increases blood flow by inducing the release of NO [2–4]. ACh has also been shown to dilate

Received on July 24, 1998; accepted on October 26, 1998

Correspondence should be addressed to: Ziya Kaygisiz, Department of Physiology, Faculty of Medicine, University of Osmangazi, 26480 Eskişehir, Turkey. Tel: +90-222-239-3750, Fax: +90-222-239-3772, E-mail: ziyak@ogu.edu.tr

isolated, precontracted epicardial coronary arteries of several species, especially the dog [5]. Furthermore, ACh exerts negative chronotropic and inotropic effects on isolated cardiac preparations [6–9]. However, other studies on perfused hearts have reported that ACh produces coronary vasoconstriction [10–12] and increases myocardial contractility [13–15]. It has been reported that positive inotropic and coronary vasoconstrictor effects of ACh on isolated rat hearts are modulated by the release of prostaglandins and NO, and both actions are muscarinic receptor mediated [15]. It is speculated that the positive inotropic effect of ACh and other muscarinic agonists in embryonic heart muscle [16] and papillar muscle of guinea pig [17] is related to the stimulation of phosphoinositide metabolism. Inositol 1,4,5-triphosphate, which is one of the products of phosphoinositide hydrolysis, is known to mobilize intracellular calcium from nonmitochondrial calcium stores in various cell types, including the sarcoplasmic reticulum [18–20]. The essential role of Ca^{2+} in the excitation and contraction processes is well known. The interaction of the contractile proteins in the myocardium is regulated by the amounts of Ca^{2+} entering the cytoplasm from extracellular fluid and the intracellular storage sites in response to membrane depolarization. These interactions of contractile proteins lead to tension development [21].

The data for understanding the underlying mechanisms of ACh-induced positive inotropy and coronary vasoconstriction remain insufficient. The possible role of calcium influx from extracellular source, the calcium release from intracellular stores, and the protein kinase C in the contractile and coronary action of ACh have not been examined. Therefore we have investigated the role of calcium influx, calcium release, and protein kinase C in the effects of ACh on contractile force, heart rate, and coronary vascular tone. We have also studied the involvement of muscarinic receptors, prostaglandins, NO, and endothelium in these cardiac dynamic effects of ACh.

METHODS

Animals were maintained according to "Guide to the Care and Use of Experimental Animals" by the Canadian Council of Animal Care [22]. Wistar rats of either sex weighing from 200 to 300 g were used in all experiments. One hour after the administration of 1,000 IU heparin i.p., the chest was opened under light ether anesthesia and the heart quickly removed, then placed in ice-cold (0 to 4°C) modified Krebs-Henseleit solution (mKHs) [23] until contractions ceased. After the heart was cleansed of surrounding fat and

other tissues, the aorta was immediately attached to a stainless steel cannula and the heart was perfused as a nonrecirculating Langendorff heart [24]. The pulmonary artery was incised to facilitate coronary drainage in ventricles. Either constant pressure (70 mmHg) or constant flow (8 ml/min) retrograde perfusion down the aorta was made. An infusion pump (Lifecare Pump, Model 4, Abbott/Shaw, Chicago) was used to perfuse hearts at a constant flow. Perfusion solution was mKHs with the following composition (mM): NaCl 118, KCl 4.7, CaCl_2 2.5, MgSO_4 1.2, KH_2PO_4 1.2, NaHCO_3 25, and glucose 11. The mKHs was continuously oxygenated with 95% O_2 and 5% CO_2 (pH 7.4) and maintained at 37°C. To measure the force of contraction, a metal hook was attached at the apex of the heart and connected to a force displacement transducer (TB 611T, Nihon Kohden, Tokyo), and a resting tension of 2 g was applied to the heart to yield optimal contractile force. Cardiac contractile force was continuously recorded on the recorder (WI 641 G, Nihon Kohden) of a polygraph (RM 6000, Nihon Kohden). The heart rate was calculated from fast-speed tracings of cardiac contraction signals. In hearts perfused under constant flow conditions, coronary perfusion pressure was measured by attaching a side arm of the aortic cannula to a pressure transducer (TP 200 T, Nihon Kohden). The coronary flow was measured in constant pressure-perfused hearts by collecting the amount of perfusate leaving the heart in a graduated cylinder. Experiments were started after a 30 min stabilization period. Drugs were infused in an aortic perfusion line at a constant rate of 0.1 ml/min by using an infusion pump (B. Braun-Melsungen AG, Bayern). The values obtained before the addition of drugs were taken as control values.

In the first part of this study, the hearts were perfused with mKHs containing ACh at doses of 10^{-7} , 5×10^{-7} , or 10^{-6} M for 10 min, then perfused with mKHs without ACh. In the second part, hearts were perfused with muscarinic receptor blocker atropine (10^{-6} M), cyclooxygenase inhibitor indomethacin (10^{-5} M), calcium channel antagonist verapamil (2×10^{-5} M), the inhibitor of release of calcium from intracellular stores dantrolene sodium (5×10^{-6} M), the inhibitor of protein kinase C staurosporine (2×10^{-6} M), or the inhibitor of EDRF (NO) synthesis N^{ω} -nitro-L-arginine methyl ester (L-NAME) (10^{-6} M) for 2 to 10 min. While cardiac dynamics were being stabilized with the agent, the hearts were being perfused with 10^{-6} M ACh for 10 min in the presence of the agent. In another group of hearts, endothelium was removed chemically by perfusing the hearts with 5% saponin solution for 30 s; then the hearts were perfused with

Fig. 1. The effects of different doses of ACh on contractile force, heart rate, and coronary flow in isolated perfused rat hearts. □, control; ▨, 10^{-7} M ACh ($n=10$); ▤, 5×10^{-7} M ACh ($n=10$); ▩, 10^{-6} M ACh ($n=10$). * Significantly different from respective control ($p < 0.001$).

10^{-6} M ACh and 5% saponin solution for 3 min.

The agents used were acetylcholine chloride (Sigma, St. Louis), L-NAME (Sigma), indomethacin (Sigma), verapamil hydrochloride (Sigma), staurosporine (Sigma), dantrolene sodium (Sigma), atropine sulfate (Haver, Istanbul), and saponin crudum (Merck, Darmstadt).

Values are given as mean ± SE. The differences among means within each group were determined by using a two-way analysis of variance. A p value of less than 0.05 was considered to be significant.

RESULTS

The peak changes in contractile force, heart rate, and coronary flow in response to different doses of ACh are shown in Fig. 1. A 10-min infusion of ACh at doses of 10^{-7} , 5×10^{-7} , and 10^{-6} M markedly increased contractile force and decreased heart rate and coronary flow. These effects of ACh were dose dependent. The control values of contractile force were

Fig. 2. The effect of ACh on contractile force in the presence of various agents. □, control; ▨, 10^{-6} M atropine ($n=8$); ▤, 10^{-6} M atropine + 10^{-6} M ACh ($n=8$); ▤, 10^{-5} M indomethacin ($n=8$); ▩, 10^{-5} M indomethacin + 10^{-6} M ACh ($n=5$); ▨, 10^{-6} M L-NAME ($n=5$); ▩, 10^{-6} M L-NAME + 10^{-6} M ACh ($n=5$). ^a Significantly different from the value in the presence of L-NAME alone ($p < 0.001$).

38.6 ± 7.46 , 41.7 ± 5.6 , and 51.1 ± 8.5 mN for 10^{-7} , 5×10^{-7} , and 10^{-6} M ACh, respectively. The contractile force increased to 52.8 ± 7.6 , 66.3 ± 6.37 , and 86.4 ± 9.74 mN for 10^{-7} , 5×10^{-7} , and 10^{-6} M ACh, respectively. ACh reduced heart rate to 39.6 ± 2.6 from 200.4 ± 16.85 , to 29.3 ± 8.83 from 230 ± 17.11 , and to 21.5 ± 6.37 from 209.5 ± 11.29 for 10^{-7} , 5×10^{-7} , and 10^{-6} M doses, respectively. ACh also reduced coronary flow to 3.9 ± 0.18 ml/min from 9.5 ± 0.62 ml/min, to 3.6 ± 0.27 ml/min from 9.45 ± 0.72 ml/min, and to 3.13 ± 0.44 ml/min from 11.13 ± 1.17 ml/min for 10^{-7} , 5×10^{-7} , and 10^{-6} M ACh, respectively. A dose of 10^{-6} M of ACh was selected to study the mechanisms of cardiac effects of ACh, since this dose was maximally effective in producing effects on mechanical and coronary functions.

The perfusion of hearts with atropine produced no significant effect on contractile force, heart rate, and coronary flow. However, the pretreatment of hearts with atropine abolished both an increase in contractile force and decreases in heart rate and coronary flow induced by ACh (Figs. 2, 4, and 6).

Indomethacin treatment had no significant effect on the baseline values of coronary flow, heart rate, and contractile force. In the presence of indomethacin, ACh also produced no significant effect on contractile force and coronary flow. However, it significantly decreased heart rate to 79 ± 15.4 from 197 ± 25.3 (Figs. 2, 4, and 6).

As shown in Figs. 2, 4, and 6, the perfusion of hearts with L-NAME induced a significant decrease in coronary flow with no significant inotropic or chronotropic effects. The administration of ACh dur-

Fig. 3. The effect of ACh on contractile force in the presence of various agents. □, control; ▨, 2×10^{-5} M verapamil ($n=6$); ▩, 2×10^{-5} M verapamil + 10^{-6} M ACh ($n=6$); ▤, 5×10^{-6} M dantrolene sodium ($n=6$); ▥, 5×10^{-6} M dantrolene sodium + 10^{-6} M ACh ($n=6$); ▦, 2×10^{-6} M staurosporine ($n=5$); ▧, 2×10^{-6} M staurosporine + 10^{-6} M ACh ($n=5$); ▨, 5% saponin ($n=10$); ▩, 5% saponin + 10^{-6} M ACh ($n=10$). * Significantly different from the control ($p < 0.001$). ^a Significantly different from the value in the presence of 5% saponin alone ($p < 0.001$).

Fig. 4. The effect of ACh on heart rate in the presence of various agents. □, control; ▨, 10^{-6} M atropine ($n=8$); ▩, 10^{-6} M atropine + 10^{-6} M ACh ($n=8$); ▤, 10^{-7} M indomethacin ($n=8$); ▥, 10^{-5} M indomethacin + 10^{-6} M ACh ($n=5$); ▦, 10^{-6} M L-NAME ($n=5$); ▧, 10^{-6} M L-NAME + 10^{-6} M ACh ($n=5$). ^a Significantly different from the value in the presence of indomethacin alone ($p < 0.001$). ^b Significantly different from the value in the presence of L-NAME alone ($p < 0.001$).

ing the perfusion of hearts with L-NAME induced a marked decrease in coronary flow to 4.5 ± 0.72 ml/min from 10.05 ± 1.32 ml/min, and heart rate to 76.7 ± 10.1 from 204 ± 18.7 . On the other hand, ACh caused a marked increase in contractile force to 78.35 ± 5.5 mN from 47.9 ± 6.7 mN.

The treatment of hearts with verapamil caused a significant decrease in contractile force and heart rate, but verapamil exerted no significant effect on coronary perfusion pressure. In hearts perfused with both verapamil and ACh, coronary perfusion pressure and contractile force were increased to 80 ± 8.65 mmHg from 52 ± 8.67 mmHg and to 49.8 ± 7.8 mN from 40.5 ± 6.48 mN, respectively. These increases were not statistically significant (Figs. 3 and 7). However, a

Fig. 5. The effect of ACh on heart rate in the presence of various agents. □, control; ▨, 2×10^{-5} M verapamil ($n=6$); ▩, 2×10^{-5} M verapamil + 10^{-6} M ACh ($n=6$); ▤, 5×10^{-6} M dantrolene sodium ($n=6$); ▥, 5×10^{-6} M dantrolene sodium + 10^{-6} M ACh ($n=6$); ▦, 2×10^{-6} M staurosporine ($n=5$); ▧, 2×10^{-6} M staurosporine + 10^{-6} M ACh ($n=5$); ▨, 5% saponin ($n=10$); ▩, 5% saponin + 10^{-6} M ACh ($n=10$). * Significantly different from control ($p < 0.001$). ^a Significantly different from the value in the presence of verapamil alone ($p < 0.001$). ^b Significantly different from the value in the presence of dantrolene sodium alone ($p < 0.001$). ^c Significantly different from the value in the presence of staurosporine alone ($p < 0.001$). ^d Significantly different from the value in the presence of 5% saponin alone ($p < 0.001$).

Fig. 6. The effect of ACh on coronary flow in the presence of different agents. □, control; ▨, 10^{-6} M atropine ($n=8$); ▩, 10^{-6} M atropine + 10^{-6} M ACh ($n=8$); ▤, 10^{-5} M indomethacin ($n=5$); ▥, 10^{-5} M indomethacin + 10^{-6} M ACh ($n=5$); ▦, 10^{-6} M L-NAME ($n=5$); ▧, 10^{-6} M L-NAME + 10^{-6} M ACh ($n=5$). * Significantly different from control ($p < 0.001$). ^a Significantly different from the value in the presence of L-NAME alone ($p < 0.001$).

marked decrease in heart rate (to 65.5 ± 11.5 from 131 ± 17.14) was observed (Fig. 5).

As illustrated in (Figs. 3, 5, and 7), dantrolene sodium exerted no significant effect on cardiac dynamics. But ACh produced a significant increase in coronary perfusion pressure and induced a marked decrease in heart rate. The coronary perfusion pressure was increased to 86.67 ± 10.2 mmHg from 60 ± 5 mmHg, and heart rate was reduced to 54.64 ± 10.8 from 215.6 ± 13.2 . ACh treatment resulted in an insignificant decline in contractile force, which was reduced to 30.3 ± 12.2 mN from 39.83 ± 8.46 mN.

Figures 3, 5, and 7 also show the influence of stau-

Fig. 7. The effect of ACh on coronary perfusion pressure in the presence of various agents. □, control; ▨, 2×10^{-5} M verapamil ($n=6$); ▤, 2×10^{-5} M verapamil + 10^{-6} M ACh ($n=6$); ▥, 5×10^{-6} M dantrolene sodium ($n=6$); ▦, 5×10^{-6} M dantrolene sodium + 10^{-6} M ACh ($n=6$); ▧, 2×10^{-6} M staurosporine ($n=5$); ▨, 2×10^{-6} M staurosporine + 10^{-6} M ACh ($n=5$); ▩, 5% saponin ($n=10$); ▪, 5% saponin + 10^{-6} M ACh ($n=10$). ^aSignificantly different from the value in the presence of dantrolene sodium alone ($p < 0.001$). ^bSignificantly different from the value in the presence of 5% saponin alone ($p < 0.001$).

rosiporine alone and ACh in the presence of staurosporine on cardiac dynamics. Staurosporine had no significant effect on coronary perfusion pressure, heart rate, and force of contraction. On the other hand, in the presence of staurosporine ACh induced a marked decrease in heart rate to 61.2 ± 8.24 from 277 ± 14 , whereas it did not significantly affect coronary perfusion pressure or force of contraction.

The perfusion of hearts with saponin caused a significant decrease in heart rate, but it did not significantly affect coronary perfusion pressure or force of contraction. Subsequent perfusion with ACh of these hearts resulted in a marked increase in force of contraction (to 152 ± 17.75 mN from 75.7 ± 7 mN) and a marked decline in coronary perfusion pressure (to 5.3 ± 0.76 mmHg from 58.2 ± 4.6 mmHg) and in heart rate (to 15.8 ± 5.31 from 104.6 ± 13.6) (Figs. 3, 5, and 7).

DISCUSSION

This study shows that the perfusion of isolated rat hearts with ACh at doses of 10^{-7} , 5×10^{-7} , and 10^{-6} M results in a marked increase in contractile force. This increase was associated with a marked decrease in coronary flow (vasoconstriction). In accordance with our results, Yang *et al.* [15] have observed with isolated rat hearts that ACh (10^{-7} , 5×10^{-7} , and 10^{-7} M) increased coronary perfusion pressure (vasoconstriction) and force of contraction.

Our data indicate that ACh can produce a positive

inotropic effect in isolated perfused rat hearts. However, our result is different from those of George *et al.* [25], who observed that ACh (7.4×10^{-8} M) produced a negative inotropic effect in isolated perfused rat hearts. This divergent response to ACh may be related to the dose of ACh used in their experiments. Furthermore, Tsuji *et al.* [16] have reported that ACh ($>10^{-6}$ M) caused a positive inotropic effect in embryonic chick ventricle, and Gilmour and Zipes [26] have reported that ACh ($>10^{-6}$ M) evoked a positive inotropic effect in canine cardiac purkinje fibers. In contrast, Ascuitto *et al.* [27] have observed that ACh (5×10^{-9} , 2×10^{-8} , and 10^{-7} M) induced a negative inotropic effect in neonatal pig hearts. Thus it appears that different findings concerning the effect of ACh on contractility may depend on the difference of doses or species, or both.

The decrease in heart rate induced by ACh may increase the diastolic filling period and lead to an increased contractile force. Therefore the increase in contractile force may depend, at least in part, on decreased heart rate. However, our results indicate that low doses of ACh (10^{-7} and 5×10^{-7} M) increased contractility. Moreover, ACh also increased contraction in hearts pretreated with L-NAME and saponin. Although we did not pace the hearts by electrical stimulation, the decrease in heart rate does not seem to be the only reason for ACh-induced increase in contractile force.

It has been observed that at low doses (10^{-6} M), ACh produced relaxation in isolated rabbit epicardial coronary arteries, whereas at higher doses ($>10^{-6}$ M), only vasoconstriction was observed [28]. In isolated donor-perfused rat hearts, Sakai [29] demonstrated that low doses of ACh decreased perfusion pressure without affecting cardiac function, though higher doses increased it. Moreover, in the guinea pig isolated perfused hearts, Stewart and Piper [30] demonstrated that low doses (10^{-10} to 10^{-8} M) of ACh elicited dose-related vasodilation. Therefore it is possible that the different effects of ACh on coronary vascular tone may be related to differences in doses. On the other hand, Lamontagne *et al.* [31] have observed that 10^{-6} M ACh induced a vasodilation in isolated perfused rabbit hearts. However, we observed that the same dose of ACh produced a vasoconstriction in isolated perfused rat hearts. This contradiction might be species related.

It is known that ACh produces vasoconstriction directly by stimulating muscarinic receptors on vascular smooth muscle [32] and indirectly through muscarinic receptors on the vascular endothelium [33]. Vasodilation is an indirect effect that results from the stimula-

tion of muscarinic receptors on endothelial cells and is mediated by NO [32]. It is possible that the population of ACh receptors causing vasoconstriction predominates over the population of ACh receptors causing vasodilation. The vasoconstriction observed in the present study is consistent with this conclusion.

Since it is known that ACh increases the K^+ conductance of sinoatrial node cells [34], the negative chronotropic effect observed in the present study can be explained by the increase of ACh-sensitive K^+ conductance in sinoatrial nodal tissue. The ACh-induced negative chronotropic effect is mediated by M_2 muscarinic receptors [34], which act via G proteins [35, 36]. When these receptors are stimulated, the special set of K^+ channels are opened and the membrane becomes hyperpolarized. Furthermore, the stimulation of M_2 muscarinic receptors produces a decrease in cyclic AMP in the cells, and this slows the opening of the Ca^{2+} channels and results in a decrease in discharge rate of sinoatrial node cells [34].

We have found that 10^{-7} , 5×10^{-7} , and 10^{-6} M ACh induced marked decreases in heart rate. Yang *et al.* [15] have shown that a high dose (10^{-6} M) of ACh produced a significant decline in heart rate, but that low doses (10^{-7} and 5×10^{-7} M) caused modest or insignificant decreases in heart rate. The perfusion duration of the hearts with ACh in the Yang's study was 4 min, whereas the hearts in our study were perfused for 10 min. Therefore this difference may be due to variations in perfusion duration with ACh.

We observed that the effect of ACh on contractile force, heart rate, and coronary flow was abolished by treatment with atropine. In support of our findings, it has been reported that the positive inotropic, negative chronotropic, and coronary vasoconstrictor effects of ACh are prevented by atropine [15, 16, 27, 29, 37–40]. Our observations with atropine suggest the conclusion that the positive inotropic, negative chronotropic, and coronary vasoconstrictor effects of ACh are related to muscarinic receptor activation.

In our experiments the pretreatment of rat hearts with indomethacin attenuated a coronary vasoconstrictor and a positive inotropic effect of ACh. Our results indicate that a cyclooxygenase product release may at least partially mediate the coronary vasoconstrictor and positive inotropic effect of ACh in isolated rat hearts. Similarly, Wagerle and Busija [41] have reported that prostanoids play an important role in cerebrovascular constrictor responses to ACh in piglets. Moreover, Myers *et al.* [42] have shown that ACh-induced vasoconstriction in isolated epicardial arteries of pig is partly mediated by a cyclooxygenase product. On the other hand, in the present study the nega-

tive chronotropic effect of ACh was not affected by indomethacin, and a marked decline in heart rate was observed. Therefore we suggest that prostaglandin release plays no significant role in the negative chronotropic effect.

It has been shown that muscarinic receptor agonist oxotremorine prolonged the duration of transmembrane action potential and produced a positive inotropic effect in the papillary muscles of guinea pig [17]. Although we have not examined the action potentials in the present study, it is possible that long-lasting ones play a role in the occurrence of ACh-induced positive inotropic effect. The extent of action potential duration results in an increase in calcium influx during action potential, and the increase in intracellular calcium is responsible for positive inotropy. On the other hand, the slow channel blocker verapamil can affect positive and negative inotropic actions of ACh. Our study shows that verapamil attenuated the coronary vasoconstrictor and positive inotropic effects of ACh. These findings are likely to be due to a reduction in the Ca^{2+} influx via slow calcium channels, and they indicate that calcium entry from extracellular space may be involved in coronary vasoconstrictor and positive inotropic response to ACh. We also found that verapamil treatment did not affect the negative chronotropic effect of ACh, indicating that the influx of extracellular calcium has no role in this effect.

We have observed that the pretreatment of rat hearts with dantrolene sodium, an inhibitor of calcium release from intracellular stores, did not alter the vasoconstrictor and negative chronotropic effects of ACh, but it did attenuate the positive inotropic effect. The blockade of the calcium release by dantrolene sodium may reduce the rate of calcium delivery to the contractile myofilaments and may be responsible for the decrease in the positive inotropic effect of ACh. It has been demonstrated that the stimulation of intracardiac nerves, which are presumed cholinergic, produces an increase in intracellular free calcium concentration [43] and a positive inotropic effect that was blocked by atropine [37, 43]. We suggest that ACh may promote the release of calcium from intracellular stores and increase the activity of intracellular calcium. The increase in intracellular calcium may at least partly account for the positive inotropic effect. Furthermore, we conclude that the calcium release from intracellular storage sites plays no role in coronary vasoconstrictor or negative chronotropic effects.

It has been reported that ACh induces the release of NO in all blood vessels and that NO produces a relaxant effect on smooth muscle. ACh has been shown to dilate precontracted rat aortic segments [44] and ca-

nine coronary arteries [45] via the release of NO. It has been observed that L-NMMA, an inhibitor of NO synthesis, increases vascular tone in rat aortic rings [46] and in human vessels [47]. In our experiments, L-NAME, an inhibitor of NO synthesis, produced a significant decline in coronary flow, suggesting that basal NO release is important in the regulation of coronary vascular tone. We have observed that ACh in the presence of L-NAME induced a marked decline in coronary flow. It is possible that the inhibition of NO release unmasked the potent vasoconstrictor action of ACh on a coronary vascular bed. We have also observed that in hearts pretreated with L-NAME, ACh caused a marked decrease in heart rate and a marked increase in contractile force. Our findings suggest that NO plays no significant role in the cardiac effects of ACh in isolated rat hearts.

In the present study, we have observed that in the presence of staurosporine, an inhibitor of protein kinase C, ACh significantly decreased heart rate, but did not significantly alter coronary perfusion pressure or force of contraction. These findings suggest that protein kinase C may mediate the positive inotropic and coronary vasoconstrictor effect of ACh, but it does not mediate the negative chronotropic effect.

Our results indicate that vasoconstrictor response to ACh depends on an intact endothelium because ACh produced no vasoconstriction after the removal of endothelial function by perfusing the hearts with saponin. Moreover, the ACh-evoked positive inotropic and negative chronotropic effects were not endothelium dependent, since the removal of endothelium did not alter these effects. Thromboxane A₂, cyclic endoperoxides, endothelin, and superoxide anions have been proposed as mediators of endothelium-dependent vasoconstriction [33]. It has been reported that a thromboxane A₂ antagonist attenuated an ACh-mediated increase in the coronary resistance of isolated rat hearts [15]. It has been suggested that at least a portion of the pressor response to ACh could occur by an endothelium-dependent mechanism [48]. Our observations suggest that a coronary vasoconstrictor effect of ACh may be mediated through the release of endothelium-derived contracting factor(s). We have also observed that ACh in the presence of saponin decreased the coronary perfusion pressure. An ACh-induced NO release may not be involved in this response because it has been demonstrated that saponin inhibited ACh-induced NO release in guinea pig isolated hearts [49]. The decrease in coronary perfusion pressure may be related to the reduction or loss of endothelium-derived contracting factor(s) after the removal of endothelium by saponin.

In conclusion, this study shows that ACh increases contractile force and decreases heart rate and coronary flow in isolated perfused rat hearts. Whereas muscarinic receptor activation is responsible for these effects of ACh, prostaglandins modulate the vasoconstrictor and positive inotropic effects of ACh. NO plays no significant role in the cardiac effects of ACh. The influx of extracellular calcium via slow channels and protein kinase C may be responsible, at least partly, for ACh-induced coronary vasoconstriction and positive inotropy. The calcium release from intracellular stores may mediate the positive inotropic effect of ACh. Furthermore, the vasoconstrictor effect of ACh depends on an intact endothelium, and the different effects of ACh on contractility and coronary vascular tone may depend on the difference of species and ACh doses.

This work was supported by the Medical and Surgical Research Center of Osmangazi University and is taken from the thesis of S. Ateş, which was submitted to the University of Osmangazi for a Ph.D. degree in physiology.

REFERENCES

1. Furchgott RF and Zawadzki JV: The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 288: 373–376, 1980
2. Cherry PD, Furchgott RF, Zawadzki JV, and Jothianandan D: The role of endothelial cells in the relaxation of isolated arteries by bradykinin. *Proc Natl Acad Sci USA* 79: 2105–2110, 1982
3. Furchgott RF: Role of the endothelium in responses of vascular smooth muscle. *Circ Res* 53: 557–573, 1983
4. Furchgott RF and Vanhoutte PM: Endothelium derived relaxing and contracting factors. *FASEB J* 3: 2007–2018, 1989
5. Kalsner S: Cholinergic mechanisms in human coronary artery preparations : implications of species differences. *J Physiol (Lond)* 358 : 509–526, 1985
6. Akahane K, Furukawa Y, Ogiwara Y, Itaniuda M, Takeda M, and Chiba S: Pharmacological analysis of chronotropic and inotropic responses to diazepam in the isolated blood perfused canine atrium. *Arch Int Pharmacodyn Ther* 290: 173–184, 1987
7. Chiba S, Karasawa Y, Adahane K, and Ken LM: Pharmacological analysis of cardiac effects of a central cholinergic agent, WEB 1881-FU, in isolated canine heart preparations. *Jpn Heart J* 290: 173–184, 1990
8. Jeck D, Lindmar R, Loffeholz K, and Wanke M: Subtypes of muscarinic receptor on cholinergic nerves and atrial cells of chicken and guinea pig hearts. *Br J Pharmacol* 93: 357–366, 1988
9. Ogiwara Y, Furukawa Y, Takeda M, and Chiba S: Blocking effects of alinidine on negative chronotropic and inotropic responses to vagal stimulation and injected acetylcholine and carbachol in dogs. *J Pharmacol Exp Ther* 243 : 1113–1120, 1987
10. Hohl CM and Hearse DJ: Vascular and contractile re-

- sponse to extracellular ATP: studies in the isolated rat heart. *Can J Cardiol* 1: 207–216, 1985
11. Karakulluğu YE, Özçelik T, and Gökhan N: Changes in cyclic nucleotides during the calcium paradox in the isolated rat heart. *Pflügers Arch* 410: 657–663, 1987
 12. Nakata T, Hearse DJ, and Curtis MJ: Are reperfusion-induced arrhythmias caused by disinhibition of an arrhythmogenic component of ischemia? *J Mol Cell Cardiol* 22: 843–858, 1990
 13. Alpoim NC, Almedia AP, Santos PE, and Freira-Maia L: Oscillations of cardiac rate induced by acetylcholine in the isolated guinea pig heart. *Braz J Med Biol Res* 22: 87–96, 1989
 14. Özçelik T, Karakulluğu YE, Unal T, and Gökhan N: Positive inotropy seen following calcium-free, acetylcholine added perfusion in cardiac muscle of the rat. *IRSC Med Sci* 13: 128, 1985
 15. Yang BC, Nichols WW, and Mehta JL: Cardiac effects of acetylcholine in rat hearts: role of endothelium-derived relaxing factor and prostaglandins. *Am J Physiol* 264: H1388–H1393, 1993
 16. Tsuji Y, Tajima T, Yuen J, and Pappano AJ: Positive inotropic effects of acetylcholine and BY K 8644 in embryonic chick ventricle. *Am J Physiol* 252: H 807–H 815, 1987
 17. Korth M and Kùhlkamp V: Muscarinic receptors mediate negative and positive inotropic effects in mammalian ventricular myocardium: differentiation by agonists. *Br J Pharmacol* 90: 81–90, 1987
 18. Hirata M, Suematsu E, Hashimoto T, Hamachi T, and Koga T: Release of Ca^{2+} from a non-mitochondrial store site in peritoneal macrophages treated with saponin by inositol 1,4,5-trisphosphate. *Biochem J* 223: 229–236, 1984
 19. Streb H, Irvine RF, Berridge MJ, and Schulz I: Release of Ca^{2+} from a non-mitochondrial intracellular store in pancreatic acinar cells by inositol 1,4,5-trisphosphate. *Nature* 306: 67–69, 1983
 20. Volpe P, Salviati G, Di Virgilio F, and Pozzan T: Inositol 1,4,5-trisphosphate induces calcium release from sarcoplasmic reticulum of skeletal muscle. *Nature* 316: 347–349, 1985
 21. Limas CJ: Calcium-binding sites in rat myocardial sarcolemma. *Arch Biochim Biophys* 179: 302–309, 1977
 22. Canadian Council on Animal Care: Guide to the Care and Use of Experimental Animals, CCAC, Ottawa, Vol 2, pp 176–187, 1984
 23. Neely JR, Libermeister H, Battersby EJ, and Morgan EH: Effect of pressure development on oxygen consumption by isolated rat hearts. *Am J Physiol* 212: 804–814, 1967
 24. Langendorff O: Untersuchungen am überlebenden Säugetierherzen. *Pflügers Arch* 61: 291–332, 1895
 25. George WJ, Wilkerson RD, and Kadowitz PJ: Influence of acetylcholine on contractile force and cyclic nucleotide levels in the isolated perfused rat heart. *J Pharmacol Exp Ther* 184: 228–235, 1973
 26. Gilmour RF Jr and Zipes DP: Positive inotropic effect of acetylcholine in canine cardiac purkinje fibers. *Am J Physiol* 249: H735–H740, 1985
 27. Ascuitto RJ, Ross-Ascuitto NT, Ramage D, McDonough KH, and Kadowitz PJ: Acetylcholine-induced coronary vasoconstriction and negative inotropy in the neonatal pig heart. *Pediatr Res* 32: 236–242, 1992
 28. Corr L, Burnstock G, and Poole-Wilson P: Responses of the rabbit epicardial coronary artery to acetylcholine and adrenoceptor agonists. *Cardiovasc Res* 25: 256–262, 1991
 29. Sakai K: Coronary vasoconstriction by locally administered acetylcholine, carbachol and bethanechol in isolated, donor-perfused, rat hearts. *Br J Pharmacol* 68: 625–632, 1980
 30. Stewart AG and Piper PJ: Vasodilator actions of acetylcholine, A 23187 and bradykinin in the guinea-pig isolated perfused heart are independent of prostacyclin. *Br J Pharmacol* 95: 379–384, 1988
 31. Lamontagne D, König A, Bassenge E, and Busse R: Prostacyclin and nitric oxide contribute to the vasodilator action of acetylcholine and bradykinin in the intact rabbit coronary bed. *J Cardiovasc Pharmacol* 20: 652–657, 1992
 32. Kalsner S: Cholinergic constriction in the general circulation and its role in coronary artery spasm. *Circ Res* 65: 237–257, 1989
 33. Lüscher TF, Boulanger CM, Dom Y, and Yang Z: Endothelium-derived contracting factors. *Hypertension* 19: 117–130, 1992
 34. Ganong WF: Review of Medical Physiology, Appleton & Lange, Norwalk, 500 pp, 1995
 35. Felder CC: Muscarinic acetylcholine receptors: signal transduction through multiple effectors. *FASEB J* 9: 619–625, 1995
 36. Hulme EC, Birdsall NMJ, and Buckley NJ: Muscarinic receptor subtypes. *Annu Rev Pharmacol Toxicol* 30: 633–673, 1990
 37. Blinks J: Field stimulation as a means of effecting the graded release of autonomic transmitters in isolated heart muscle. *J Pharmacol Exp Ther* 151: 221–235, 1966
 38. Hollenberg M, Carriere S, and Barger AC: Biphasic action of acetylcholine on ventricular myocardium. *Circ Res* 7: 527–536, 1965
 39. Inui J, Brodde OE, and Schümann HJ: Influence of acetylcholine on the positive inotropic effect evoked by α or β -adrenoceptor stimulation in the rabbit heart. *Naunyn-Schmiedeberg's Arch Pharmacol* 320: 152–159, 1982
 40. Korth M and Kùhlkamp V: Muscarinic receptor-mediated increase of intracellular Na^+ -ion activity and force of contraction. *Pflügers Arch* 403: 266–272, 1985
 41. Wagerle LC and Busija DW: Effect of thromboxane A_2 /endoperoxide antagonist SQ 29548 on the contractile response to acetylcholine in newborn piglet cerebral arteries. *Circ Res* 66: 824–831, 1990
 42. Myers PR, Banitt PF, Guerra R, and Harrison DG: Role of the endothelium in modulation of the acetylcholine vasoconstrictor response in porcine coronary microvessels. *Circ Res* 25: 129–137, 1991
 43. Endoh M and Blinks JR: Effect of endogenous neurotransmitters on calcium transients in mammalian atrial muscle. *In: Neuronal and Extraneuronal Events in Autonomic Pharmacology*, ed. Fleming WW, Graefe KH, Langer SZ, and Weiner N, Raven, New York, pp 221–230, 1984

44. Yang BC, Lawso DL, Nichols WW, Mehta P, and Mehta JL: Agonist-induced tension determines reactivity of rat aortic rings during anoxia and reoxygenation. *Life Sci* 50: 1805–1812, 1992
45. Lawson DL, Mehta JL, and Nichols WW: Coronary reperfusion in dogs inhibits endothelium-dependent relaxation: role of superoxide radicals. *Free Radical Biol Med* 8: 373–380, 1990
46. Mehta JL, Lawson DL, Yang BC, Haught WH, and Hintze T: Role of superoxide radicals in anoxia and reoxygenation-mediated vascular contraction. *Life Sci* 49: 1739–1746, 1991
47. Marin J and Sanchez Ferrer CF: Role of endothelium-formed nitric oxide on vascular responses. *Gen Pharmacol* 21: 575–587, 1990
48. Hoover DB and Neely DA: Differentiation of muscarinic receptors mediating negative chronotropic and vasoconstrictor responses to acetylcholine in isolated rat hearts. *J Pharmacol Exp Ther* 282 : 1337–1344, 1997
49. Ellwood AJ and Curtis MJ : Mechanism of 5-hydroxytryptamine-induced coronary vasodilation assessed by direct detection of nitric oxide production in guinea-pig isolated heart. *Br J Pharmacol* 119: 721–729, 1996