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Acetylcholine-Induced Coronary Vasoconstriction and Negative Inotropy in the Neonatal Pig Heart

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ABSTRACT. We investigated the influence of exogenously administered acetylcholine, nitric oxide, ADP, ATP, bradykinin, and substance P on coronary vascular tone in isolated, neonatal pig hearts (≤4 d). Paced (180 bpm), isovolumically beating hearts underwent retrograde aortic perfusion, with an erythrocyte-enriched solution (hematocrit 0.15–0.20) at constant coronary flow (≈2.5 mL/min/g) corresponding to a perfusion pressure of ≈60 mm Hg. Agonists were injected into the aortic root, and the peak change in coronary perfusion pressure from baseline and left ventricular pressure development were assessed. Nitric oxide (3 μL), ADP (30 nmol), ATP (30 nmol), bradykinin (125 ng), and substance P (50 ng) decreased the perfusion pressure (vasodilatation) by 16.9 ± 1.2, 25.3 ± 4.4, 18.3 ± 1.2, 18.9 ± 1.4, and 7.1 ± 1.6 mm Hg, respectively. Acetylcholine (0.5 and 1.0 nmol) produced a modest decrease in perfusion pressure (vasodilatation) of 4.2 ± 0.8 and 3.8 ± 0.5 mm Hg, respectively, whereas acetylcholine (5, 20, and 100 nmol) increased the perfusion pressure (vasoconstriction) by 16.7 ± 2.7, 48.2 ± 8.2, and 85.3 ± 15.1 mm Hg, respectively. Acetylcholine also decreased left ventricular peak systolic pressure from 108.7 ± 5.0 to 69.2 ± 4.6, 56.3 ± 6.1, and 48.2 ± 6.4 mm Hg, for the 5, 20, and 100 nmol doses, respectively. Responses to acetylcholine were abolished by atropine (50 nmol). In a separate group of hearts, indomethacin (10^{-6} M) reduced the peak change in perfusion pressure for the 5, 20, and 100 nmol doses of acetylcholine by 87%, 66%, and 48%, respectively. In other hearts, the calcium channel antagonist, nisoldipine (10^{-7} M), reduced the peak change in perfusion pressure for the 5, 20, and 100 nmol doses of acetylcholine by 87%, 77%, and 56%, respectively. In summary, acetylcholine causes predominantly coronary vasoconstrictive and negative inotropic effects in neonatal pig hearts; both actions are muscarinic receptor mediated. Our data also indicate that a cyclooxygenase product may, in part, be involved in this vasoconstriction, and that an extracellular source of calcium contributes to the vasoconstrictive process. (*Pediatr Res* 32: 236–242, 1992)

Abbreviations

ACh, acetylcholine
EDRF, endothelium-derived relaxing factor
BK, bradykinin
sub P, substance P
NO, nitric oxide
PSP, peak systolic pressure
dP/dt, change in pressure per unit time

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Some 30 years ago, Cassin *et al.* (1) showed that ACh dilates the normally high tone pulmonary vasculature of the fetus. More recently, ACh has been shown to be a potent dilator of precontracted, isolated systemic and pulmonary arteries from neonates of several species (2). As originally demonstrated by Furchgott and Zawadzki (3), the vasorelaxation produced by ACh requires the presence of a functionally intact endothelium and involves the production of an EDRF. ACh has also been shown to relax precontracted, epicardial coronary arteries isolated from several species, most notably the dog (4). It has become apparent, however, that these results cannot be generalized to all mammals, because large coronary arteries of adult pigs (5) and humans (6, 7) can constrict *in vitro* to exogenously administered ACh. Recently, Cowan and McKenzie (8) demonstrated that ACh reduces myocardial blood flow when injected into the left anterior descending coronary artery of *in vivo* adult pig hearts. Although this finding is consistent with the vasoconstrictor responses observed with ACh using isolated coronary arteries, the coronary vascular effects of ACh in intact, functioning hearts require additional investigation. Such information is of importance, because cholinergic mechanisms may play a role in the regulation of coronary blood flow. Moreover, little, if anything, is known about the actions of ACh on the coronary vasculature of the immature heart.

Therefore, the purpose of the present investigation was to study the effects of ACh on mechanical function and coronary vascular tone, using neonatal pig hearts. Isolated, isovolumically beating hearts were perfused with an erythrocyte-enriched solution under conditions of constant coronary flow. The effects of NO, ADP, ATP, BK, and sub P on the coronary vasculature were also examined. NO, which is believed to be EDRF, was studied as an example of a direct smooth muscle vasodilator (9). The remaining agents were investigated because they produce endothelial-dependent coronary vasodilatation in mature hearts (10). We hypothesized that ACh would cause coronary vasoconstriction in neonatal pig hearts, and that this vasoconstriction would be inhibited by indomethacin, because ACh has also been shown to produce cerebrovascular constriction in piglets, which is mediated by the release of cyclooxygenase products (11).

MATERIALS AND METHODS

Preparation of erythrocytes and perfusate. The perfusate (37°C) consisted of a crystalloid solution containing: 200 μU/mL insulin, 118 mM NaCl, 4.7 mM KCl, 2.4 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.4 mM $CaCl_2$, 5.5 mM glucose, 2–

4 mM lactate, and 2% BSA (Cohn Fraction V; U.S. Biochemical Corp., Cleveland, OH). Outdated, saline-washed, human erythrocytes (tested negative for hepatitis B and human immunodeficiency virus) were then suspended in this solution (hematocrits 0.15–0.20) to provide adequate oxygen delivery and buffering capacity for these large hearts (12.6 ± 1.3 g). The pH of the final solution was adjusted to 7.4–7.45 by titration with 1 N NaOH.

Preparation of solutions. Indomethacin (Sigma Chemical Co., St. Louis, MO) was dissolved in warm $NaCO_3$ (0.08%) and protected from light. An aliquot of this solution was added to the perfusate to yield an indomethacin concentration of 10^{-6} M. Nisoldipine (Miles Inc., Dallas, TX) was dissolved in Cremophor EL (Sigma), Tris, and Tris-HCl (pH 7.4). The resulting suspension was warmed, and 2 mL of polyethylene glycol and 10 mL of Tris (pH 7.4) were added. This stock solution was stored frozen and protected from light. When required, this solution was thawed, diluted with saline, and infused into the aortic root at a rate equivalent to a perfusate nisoldipine concentration of 10^{-7} M. ACh (Sigma), ADP, and ATP (Boehringer Mannheim, Indianapolis, IN) were dissolved in saline and prepared on the day of an experiment. BK (Sigma) and sub P (Sigma) were also dissolved in saline and frozen (≈−10°C) in bottles protected from light. Stock solutions were thawed for each experiment and diluted in saline when necessary. NO was dissolved in reagent grade methanol under O_2-free conditions. The precise concentration of NO in the solution was unknown, although it was estimated to be 1–2 mM. Thus, only the volume of the NO solution injected is specified. Atropine sulfate (Abbott Laboratories, Chicago, IL) was dissolved in perfusate solution and injected as a bolus into the aortic root.

Preparation of isolated hearts. Pigs, 12 h to 4 d of age, were used for these studies. Isolated, isovolumically beating hearts were prepared as described previously (12, 13). Briefly, piglets were anesthetized with sodium pentobarbital (Nembutal, 25 mg/kg intraperitoneally; Abbott), anticoagulated with sodium heparin (heparin, 1000 U i.v.), and mechanically ventilated via a tracheostomy. After a sternotomy, the hearts were excised rapidly and immediately placed in ice-cold, Krebs-Henseleit solution.

Using the modified Langendorff system described previously (12, 13) and depicted in Figure 1, nonrecirculating, retrograde-aortic perfusion was initiated with the crystalloid solution for ≈5 min (to flush the coronary arteries of debris), and then continued with the erythrocyte-enriched solution. Hearts were allowed to equilibrate while being perfused for ≈15 min at constant coronary perfusion pressure. The enhanced oxygen-carrying capacity provided by the erythrocytes ensured that coronary flow rates could be kept in the physiologic range (14) without compromising mechanical function. Furthermore, coronary vascular tone would be maintained and coronary vasodilator responses could be elicited during the course of an experiment. We have demonstrated previously for example, that these piglet hearts exhibit hyperemic responses after ischemia (12) and that coronary vascular resistance can decrease ≈3-fold with hypoxia (13). Thus, the coronary vascular reserve was adequate, and responses to agonists could be studied without the necessity, and additional complexity, of using agents to increase or decrease coronary vascular tone. Such agents might also adversely affect mechanical function.

After equilibration, hearts were then perfused at constant coronary flow (≈2.5 mL/min/g wet heart wt) that was chosen to produce a coronary perfusion pressure of ≈60 mm Hg. Perfusing hearts at constant coronary flow allowed us to study vasoconstrictor responses without impairing oxygen delivery, which may have compromised mechanical function. Also, because coronary flow was held constant, changes in coronary perfusion pressure reflected alterations in coronary vascular tone. In this preparation, coronary venous return egressed from the pulmonary artery cannula, from which coronary flow was measured. Left ventricular mechanical function was assessed by isovolumic systolic pressure and the maximum rate of change of systolic pressure

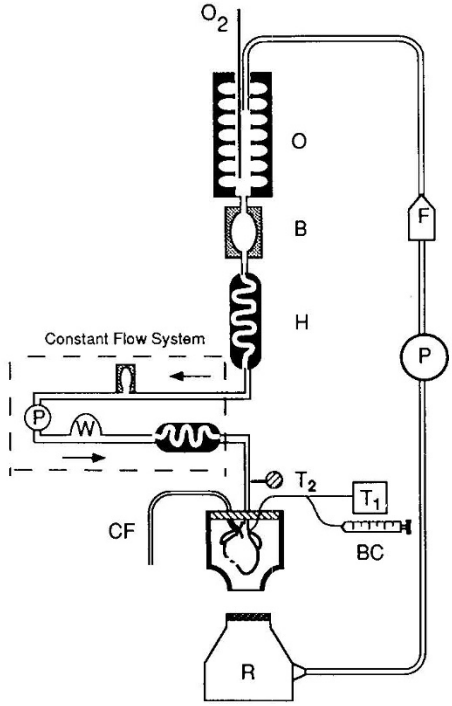

Fig. 1. Perfusate in reservoir (*R*) was pumped through an oxygenating column (*O*), bubble trap (*B*), and heating condenser (*H*). Before entering the aorta, the perfusate passed through a constant-flow system containing a pump (*P*) and a pulsatile flow-damping chamber (*W*), which maintained the coronary flow at the desired constant value. Myocardial temperature was kept at 37°C by enclosing the heart in a heated water jacket. Left ventricular isovolumic pressure was monitored by a pressure transducer (T_1) attached to a fluid-filled balloon catheter (*BC*) in the left ventricular chamber. Coronary perfusion pressure was monitored with a pressure transducer (T_2) attached to a side port above the heart. Coronary venous return egressed from the pulmonary artery cannula (*CF*). Coronary venous return was collected separately and not returned to the reservoir (*R*).

with respect to time (dP/dt), which were monitored via a fluid-filled balloon catheter in the ventricular chamber. The left ventricular end diastolic pressure was adjusted by changing the volume of fluid in the balloon. The end diastolic pressure was set at 2–4 mm Hg, which is normal for a neonate. Hearts were paced via the right ventricle at 180 bpm, so that ventricular contractions could be maintained at a constant rate when studying agents (ACh) that affect atrioventricular conduction. The arterial perfusate oxygen content was maintained at ≈7.5–8.0 vol% [Po_2 ≈250–300 torr (33.3–40 kPa)] by passing a mixture of 95% O_2 and 5% CO_2 through the perfusate in the Langendorff column.

Experimental protocols. The first part of this study was designed to test whether ACh produces coronary vasoconstriction in neonatal pig hearts. Hearts (n = 5) underwent retrograde aortic perfusion at constant coronary flow. The agonists ACh (5, 20, and 100 nmol), ADP (30 nmol), BK (125 ng), and sub P (50 ng) were injected as a bolus, over an ≈1-s period, into the aortic root of hearts in a random sequence. The resulting changes in coronary perfusion pressure from baseline (*i.e.* just before the administration of each agonist) were then determined. Changes in perfusion pressure were expressed in mm Hg. Agonists were administered only after perfusion pressure had stabilized. Based on preliminary studies, the doses of agonists were selected so that vascular tone would return to near control levels upon washout of the agonist. These doses were also found to be similar to those used in previous vascular studies (10, 15–18). In additional hearts, NO (3 μL; n = 5) and ATP (30 nmol; n = 5) were studied in a similar fashion. These experiments were performed to verify

that the coronary vasculature of these isolated hearts responded to a direct smooth muscle vasodilator (NO) in conjunction with an endothelial dependent vasodilator (ATP).

The second part of this investigation was designed to determine whether the release of a cyclooxygenase product mediates the vasoconstrictive effect of ACh. The three doses of ACh were administered to five additional hearts in amounts of 5, 20, and 100 nmol, and the corresponding changes in perfusion pressure were determined. Alterations in left ventricular mechanical function in response to ACh were also assessed. After baseline measurements, hearts were perfused for an additional 30 min with perfusate supplemented with indomethacin (10^{-6} M). Hearts were then rechallenged with ACh in the presence of indomethacin. This concentration of indomethacin has routinely been used by other investigators in studies of vascular responses to arachidonic acid, ACh, and BK (2, 15). The peak changes in perfusion pressure in response to ACh, in the presence of indomethacin, were compared with the corresponding changes in perfusion pressure obtained with the same doses of ACh, but before the addition of indomethacin. The peak changes in perfusion pressure in response to the doses of ACh, in the presence of indomethacin, were also compared with corresponding changes in perfusion pressure obtained in four control hearts perfused for the same period of time but without the addition of indomethacin. Because indomethacin was found to increase perfusion pressure and, thus, vascular tone, ACh was also studied in three additional hearts in which the vasoconstricting thromboxane analogue U46619 was injected as a bolus into the aortic root before the administration of ACh. On average, 10 ng of U46619 was found to increase coronary perfusion pressure ≈5 mm Hg. We chose a dose of U46619 so as to increase perfusion pressure ≈25 mm Hg, the increase in perfusion pressure found with indomethacin (see Results). Thus, these experiments served as an additional control for the indomethacin studies and also allowed us to investigate whether an increased vascular tone unmasked a vasodilation from ACh.

The third part of this study examined whether the vasoconstriction caused by ACh involved an extracellular source of calcium. ACh was administered to five additional hearts, and the changes in perfusion pressure were determined. After baseline measurements, hearts were perfused for an additional 20 min with the dihydropyridine calcium antagonist, nisoldipine, which was infused into the aortic root at a rate equivalent to a perfusate concentration of 10^{-7} M. Hearts were then rechallenged with ACh in the presence of nisoldipine. This concentration of nisoldipine has been shown to inhibit ACh-induced contractions of adult pig epicardial coronary artery strips (19). The peak changes in perfusion pressure in response to ACh, in the presence of nisoldipine, were compared with the corresponding changes in perfusion pressure obtained before the addition of nisoldipine.

Data analysis. The results are presented as mean ± SEM. Analysis of variance with repeated measures followed by the Student-Newmann-Keuls comparison procedure was used to analyze differences in the response to ACh or other agonists. Differences among means were considered to be significant when $p < 0.05$.

RESULTS

Influence of ACh and agonists on coronary perfusion pressure. The peak change in coronary perfusion pressure, in response to each vasoactive agonist, is shown in Figure 2. In these experiments, coronary flow was 2.6 ± 0.3 mL/min/g, which corresponded to the coronary perfusion pressures shown in Table 1. NO, ADP, ATP, BK, and sub P, as expected, decreased perfusion pressure, indicating coronary vasodilatation. In contrast, ACh produced dose-dependent increases in perfusion pressure, signifying vasoconstriction. The vasoconstrictive response to ACh was abolished by pretreatment with bolus injections into the aortic root of the muscarinic receptor antagonist, atropine (50 nmol). The bolus administration of atropine itself had no effect on perfusion pressure or mechanical function.

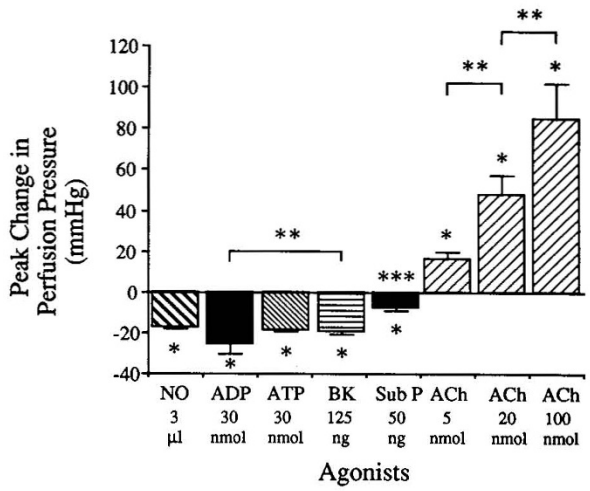

Fig. 2. Peak change in coronary perfusion pressure, expressed in mm Hg, in response to the agonists NO (3 µL), ADP (30 nmol), ATP (30 nmol), BK (125 ng), sub P (50 ng), and ACh (5, 20, and 100 nmol). The results are expressed as mean ± SEM in all figures. *Asterisks* indicate statistically significant differences from baseline coronary perfusion pressure, *i.e.* perfusion pressure just before the addition of each agonist. *Asterisks above the horizontal lines* indicate statistically significant differences between the groups joined by the lines. ***, the response to sub P is statistically different when compared with NO, ADP, ATP, and BK.

Table 1. *Coronary perfusion pressures before administration of agonists*

Agonist	Dose	Perfusion pressure*
NO	3 µL	61.6 ± 2.5
ADP	30 nmol	60.5 ± 3.0
ATP	30 nmol	63.3 ± 3.4
BK	125 ng	58.1 ± 1.6
Sub P	50 ng	56.3 ± 6.3
ACh	5 nmol	50.5 ± 3.8
ACh	20 nmol	53.5 ± 3.5
ACh	100 nmol	56.0 ± 2.7

* Baseline coronary perfusion pressures (mm Hg), mean ± SEM, immediately before the administration of the agonists indicated.

Influence of ACh on mechanical function. Under baseline conditions, ACh produced dose-dependent increases in coronary perfusion pressure and decreases in left ventricular PSP, as shown in Figure 3. The negative inotropic effect was also abolished by pretreatment with atropine. Figure 4 shows a typical tracing for perfusion pressure, left ventricular pressure, and dP/dt in response to 20 nmol of ACh. Upon administration of ACh, there was a dramatic increase in perfusion pressure and a reduction in left ventricular systolic pressure and dP/dt. Similar effects on left ventricular pressure were found for the 5- and 100-nmol doses of ACh. The duration of action of ACh was short (≈1 min), and there was a rebound in left ventricular systolic pressure and a slight decrease in perfusion pressure, below baseline (vasodilatation), after washout of ACh. NO, ADP, ATP, BK, and sub P were found to produce only minor decreases in left ventricular PSP, averaging ≈5 mm Hg (data not shown).

Influence of indomethacin on responses to ACh. In these experiments, coronary flow was 2.3 ± 0.1 mL/min/g. The cyclooxygenase inhibitor, indomethacin (10^{-6} M), increased the baseline coronary perfusion pressure by 24 ± 7 mm Hg in three hearts, whereas in the other two hearts perfusion pressure increased transiently. Indomethacin also reduced the peak change in perfusion pressure for the 5-, 20-, and 100-nmol doses of ACh by 87%, 66%, and 48%, respectively (Fig. 5). Indomethacin was

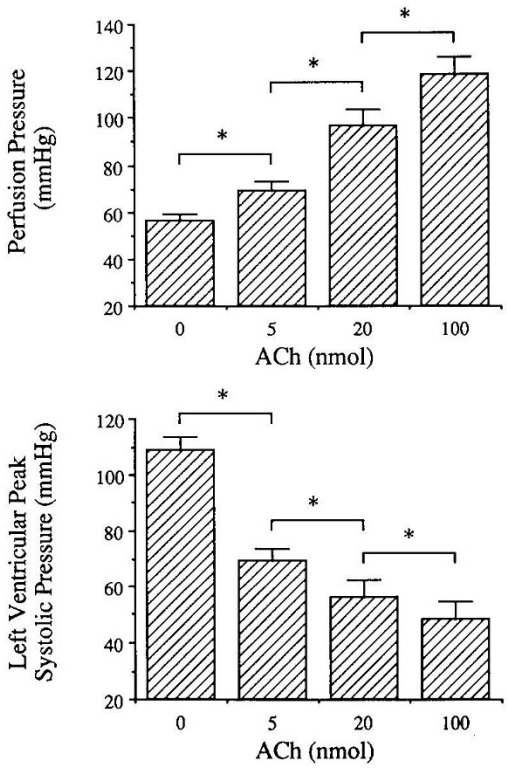

Fig. 3. The *top graph* demonstrates the effects on coronary perfusion pressure, expressed in mm Hg, in response to ACh (5, 20, and 100 nmol). The *bottom graph* shows the effects on left ventricular PSP, expressed in mm Hg, in response to the three doses of ACh. *Asterisks* indicate statistically significant differences as described above.

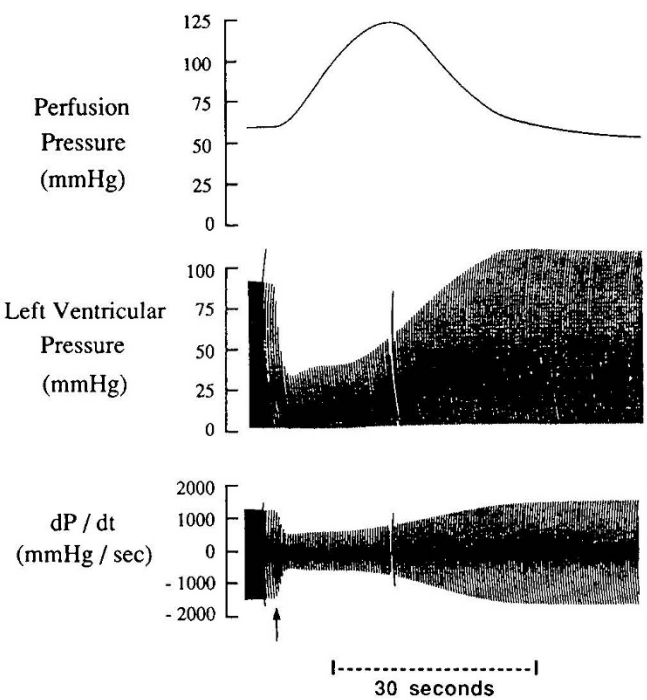

Fig. 4. An example of a tracing illustrating the responses with respect to time to a 20-nmol dose of ACh. Perfusion pressure (*top*) and left ventricular pressure (*middle*) are expressed in mm Hg; dP/dt (*bottom*) is expressed in mm Hg/s. The *arrow* indicates when ACh was administered.

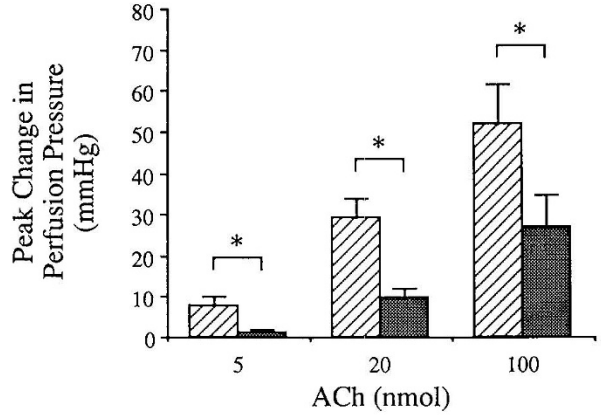

Fig. 5. Peak changes in perfusion pressure, expressed in mm Hg, during baseline conditions (▨) and in the presence of indomethacin (▩, 10^{-6} M) in response to the three doses of ACh. *Asterisks* indicate statistically significant differences.

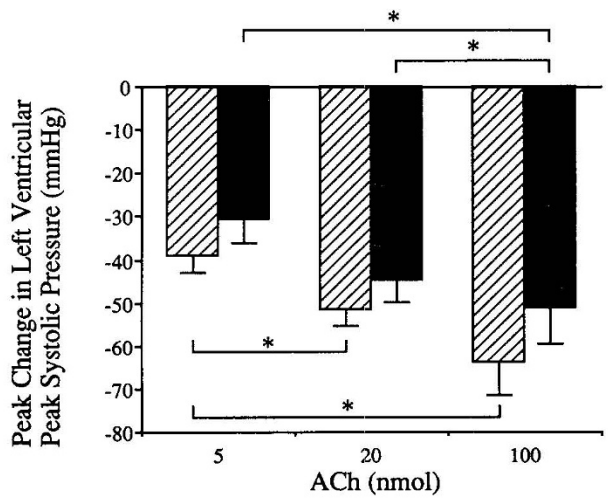

Fig. 6. Peak changes in left ventricular PSP, expressed in mm Hg, in response to the three doses of ACh (▨) and to the three doses of ACh administered in the presence of indomethacin (■, 10^{-6} M). *Asterisks* indicate statistically significant differences as described above.

also found to reduce the magnitude of the decrease in left ventricular PSP, produced by the 5-, 20-, and 100-nmol doses of ACh, on average by ≈22%, ≈13%, and ≈20%, respectively (Fig. 6). The baseline PSP, before the administration of ACh and the addition of indomethacin, was 103.3 ± 4.0 mm Hg; the PSP, when measured ≈44 min later, before rechallenging with ACh and still in the presence of indomethacin, was 99.5 ± 4.4 mm Hg. These results indicate that, although indomethacin had little effect on PSP, indomethacin still had a significant effect on perfusion pressure. In four control hearts, perfused for an equivalent period of time but without the addition of indomethacin, there was no substantial decrease over time in the vasoconstrictor response to ACh (Fig. 7). In these control hearts, the baseline PSP, before the administration of ACh, was 101.3 ± 1.3 mm Hg; the PSP when measured ≈42 min later, before rechallenging with ACh, was 96.7 ± 1.7 mm Hg.

The influence of increasing coronary perfusion pressure with U46619 on the responses to ACh is shown in Figure 8. At elevated tone, the vasoconstrictive response to ACh was slightly attenuated, although this effect is not statistically significant. Furthermore, when one compares the responses to ACh in the presence of indomethacin (Fig. 5) to those in the presence of U46619 (Fig. 8), it can be seen that indomethacin's attenuation of ACh's vasoconstrictive response is not due simply to indomethacin's effect on vascular tone. In these experiments, we also

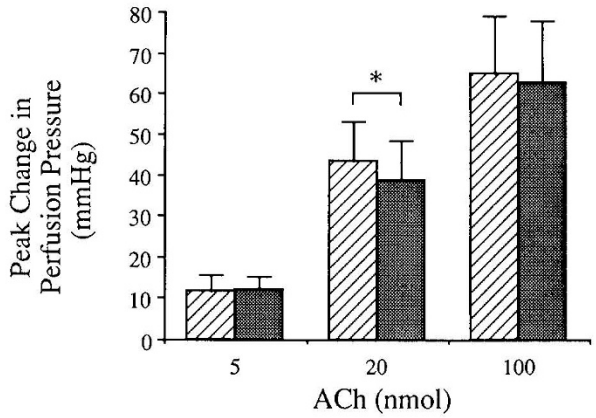

Fig. 7. Peak changes in perfusion pressure, in mm Hg, in control hearts during baseline conditions (▨) and after an additional ≈45 min of perfusion (▩) in response to the three doses of ACh. For the 20-nmol dose of ACh, there was a statistically significant difference (*asterisk*) in the peak change in perfusion pressure over time; however, this decrease in perfusion pressure was inconsequential when compared with the decrease observed with indomethacin (see Fig. 5).

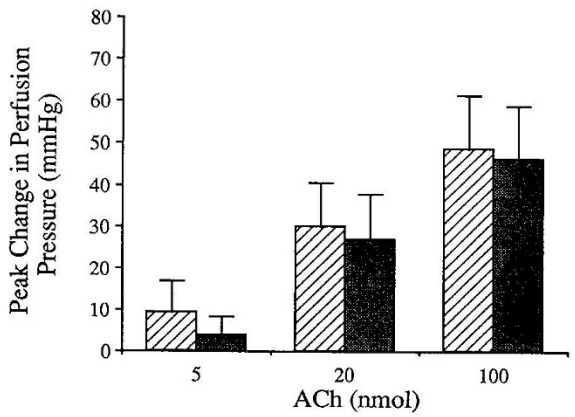

Fig. 8. Peak changes in perfusion pressure, expressed in mm Hg, during baseline conditions (▨) and in the presence of U46619 (▩) in response to the three doses of ACh. No statistically significant differences were found.

found that the coronary vasoconstriction produced by U46619 can result in a small transient decrease in PSP. On average, a single bolus of U46619 that increases perfusion pressure ≈25 mm Hg resulted in an ≈7 mm Hg decrease in PSP.

Influence of nisoldipine on vasoconstrictor responses to ACh. In these experiments, coronary flow was 2.9 ± 0.2 mL/min/g. The dihydropyridine calcium antagonist, nisoldipine (10^{-7} M), decreased the baseline coronary perfusion pressure by 16 ± 4 mm Hg. Nisoldipine also reduced the peak change in perfusion pressure for the 5-, 20-, and 100-nmol doses of ACh by 87%, 77%, and 56%, respectively (Fig. 9).

Influence of age on vasoconstrictor responses to ACh. Our experiments also suggest that there may be a maturational change in the vasoconstrictor response to ACh, because hearts from older animals showed greater vasoconstrictor responses. Table 2 illustrates this by comparing the peak changes in perfusion pressure, in response to ACh, for hearts from the two youngest (12–36 h) and the two oldest (72–96 h) pigs. Furthermore, if the dose of ACh was administered on the basis of heart weight, the blunted vasoconstrictive response in the younger hearts would become even more pronounced.

Vasodilator responses to ACh. In nine of the 19 experiments, ACh was observed to cause a slight decrease in perfusion pressure (≈1.5 mm Hg), before the increase in perfusion pressure. This effect was most evident at the 5-nmol dose, and was more

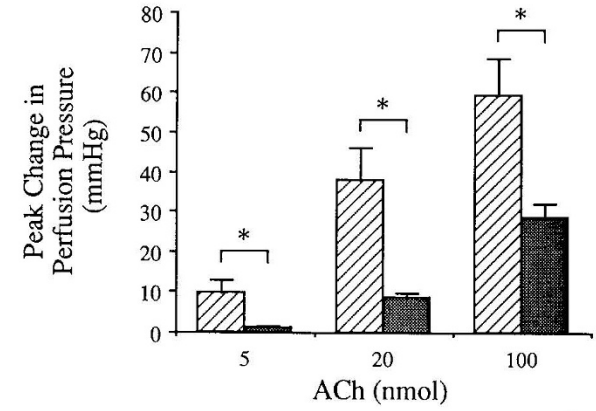

Fig. 9. Peak changes in perfusion pressure, expressed in mm Hg, during baseline conditions (▨) and in the presence of nisoldipine (▩, 10^{-6} M) in response to the three doses of ACh. *Asterisks* indicate statistically significant differences.

Table 2. *Response in perfusion pressure to ACh in youngest and oldest piglets**

	Youngest piglets ($n = 2$)		Oldest piglets ($n = 2$)	
Heart wt (g)	7.4	10.7	15.7	23.8
Change in PP, 5 nmol ACh	15.0	10.5	20.0	20.0
Change in PP, 20 nmol ACh	27.0	28.8	61.3	58.8
Change in PP, 100 nmol ACh	35.0	40.0	101.3	120.0

* Comparison of peak change in coronary perfusion pressure (PP, mm Hg) to ACh between youngest (12–36 h) and oldest (72–96 h) piglets.

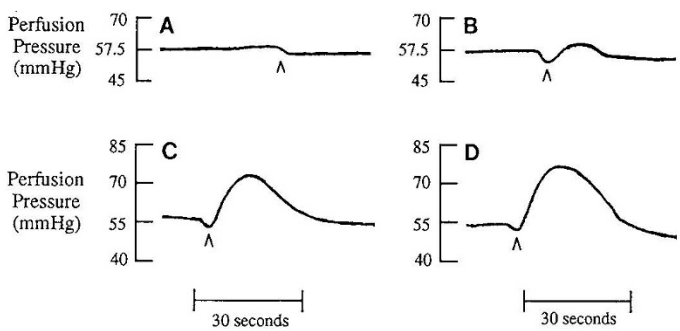

Fig. 10. An example of a tracing illustrating the change in perfusion pressure, expressed in mm Hg, with respect to time to 1-nmol (*A*) 5-nmol (*B*) 20-nmol (*C*), and 100-nmol (*D*) doses of ACh. *Arrow* indicates the vasodilatory response within several seconds after administration of ACh.

pronounced for hearts from the youngest animals (Fig. 10). We verified that the decrease in perfusion pressure was not simply related to the vehicle or the mode of delivery of ACh. This observation prompted us to study the effects of lower doses of ACh, *i.e.* 0.5 and 1.0 nmol. ACh reduced perfusion pressure from 62.1 ± 1.1 to 57.9 ± 0.4 mm Hg ($n = 3$) and from 59.7 ± 1.3 to 56.0 ± 0.9 mm Hg ($n = 4$) for the 0.5- and 1.0-nmol doses, respectively. Of interest was that these decreases in perfusion pressure were also associated with a negative inotropic effect.

DISCUSSION

In the present study, we observed coronary vasodilatation in response to NO and to ADP, ATP, BK, and sub P in neonatal pig hearts, which is consistent with smooth muscle that is re-

sponsive and an endothelium that is functionally intact and developed. This study also demonstrated that exogenously administered ACh produced dose-dependent coronary vasoconstriction. This vasoconstriction was abolished by atropine, and thus is muscarinic receptor mediated. In support of our findings, recent studies have shown that ACh contracts large coronary arteries isolated from adult pigs (5) and humans (6). The mechanism by which ACh causes this vasoconstriction, however, has not been delineated. Graser *et al.* (20) have suggested that the vasoconstriction produced by ACh in the left circumflex coronary artery of the pig does not require the presence of an intact endothelium. Similarly, Toda (21) has demonstrated an endothelium-independent, ACh-induced contraction of human coronary artery preparations. These experiments suggest that ACh may cross the endothelium to act directly on muscarinic receptors on the vascular smooth muscle. In contrast, Myers *et al.* (22) have shown that an ACh-induced contraction in large coronary arteries of pigs is augmented by agents that inactivate or inhibit the synthesis of EDRF. However, Myers *et al.* (22) and Christie *et al.* (23) found no evidence for ACh-stimulated EDRF release, although EDRF was not actually measured. These investigations would suggest that EDRF was only involved in the regulation of basal coronary artery tone. Therefore, it appears that the coronary vasoconstrictor response to ACh may not only involve a direct influence of ACh on vascular smooth muscle, but may also be opposed by an EDRF-induced vasodilatation.

In the functioning, neonatal pig heart, we found that indomethacin (10^{-6} M) attenuated the coronary vasoconstrictor response to ACh by between 48% and 87%, suggesting that cyclooxygenase product release may at least partially mediate this response. Thromboxane A_2 or prostaglandin endoperoxide could be involved in the vasoconstrictor process (24), inasmuch as Wagerle and Busija (25) have shown recently that these prostanoids play an important role in cerebrovascular constrictor responses to ACh in piglets. Moreover, Myers *et al.* (22) have shown that ACh's vasoconstriction in isolated, epicardial coronary arteries of the pig is, in part, mediated by a cyclooxygenase product, inasmuch as the vasoconstriction is attenuated by indomethacin. Our results are consistent with these findings, although our whole heart preparation does not allow us to distinguish which coronary artery segments may be more or less responsive to the effects of indomethacin. Furthermore, to demonstrate whether the vasoconstrictor response to ACh (or the inhibition of this constriction by indomethacin) depends upon an intact endothelium, responses to ACh would have to be studied in piglet hearts that have undergone disruption of the endothelium, *e.g.* by perfusing hearts with saponin (26). These experiments are beyond the scope of the present investigation.

Our study also showed that the dihydropyridine calcium antagonist, nisoldipine, attenuated the coronary vasoconstrictor response to ACh, indicating that calcium influx from the extracellular space was involved in the smooth muscle contractile process. Endothelium-dependent contracting factors have been described that are cyclooxygenase products and that exert their effects through transsarcolemmal calcium influx (27). Therefore, one cannot exclude the possibility that the coronary vasoconstrictor response to ACh, in these neonatal pig hearts, may involve the release of an endothelium-derived contracting factor.

ACh was also found to produce a dose-dependent negative inotropic effect in neonatal pig hearts that was also muscarinic receptor mediated. ACh is known to have negative inotropic effects in atria (28); however, the action of ACh in ventricular myocardium has not been well characterized. McIvor *et al.* (29) and Boyett *et al.* (30) have shown that ACh reduces developed tension in adult ferret papillary muscle. These investigators have suggested that the negative inotropic effect of ACh is a consequence of a shortening of the action potential duration, which results in a decrease in calcium influx. To our knowledge, ACh's effect on action potential duration has not been examined in neonatal hearts.

The coronary vasoconstriction produced by ACh could conceivably contribute to the negative inotropic effect. Our hearts were perfused at constant coronary flow, and thus it is unlikely that vasoconstriction impaired myocardial oxygen delivery. Nevertheless, coronary vasoconstriction to ACh may be heterogenous (6, 21), *e.g.* in regard to responses in epicardial *versus* endocardial vessels. Such an effect could result in redistribution of flow and in regions of myocardial underperfusion, and, thus, impaired mechanical function. The slight coronary vasodilatation observed during washout of ACh (Fig. 4) may reflect a release of local metabolites associated with regional underperfusion. Coronary vasoconstriction, however, cannot be a major determinant of the negative inotropic response, because ACh continued to cause a significant decrease in peak systolic pressure, even at lower doses (*e.g.* 0.1 nmol), for which we observed either no effect on perfusion pressure (data not shown) or a reduction in perfusion pressure (see Results). Additionally, preconstriction of the coronary vasculature, with a bolus of U46619, to a level comparable to that produced by the 5-nmol dose of ACh resulted in only a minimal decrease in peak systolic pressure. It is plausible, however, that a cyclooxygenase product may have participated in the negative inotropic effect, because the decrease in left ventricular PSP produced by ACh was attenuated (by $\approx$18%) in the presence of indomethacin. Although our study demonstrated that systolic pressure development was significantly reduced by ACh, it is not known whether the parasympathetic innervation is sufficiently developed in neonatal hearts for the negative inotropic effect to be physiologically important.

Feigl *et al.* (31) studied ACh in *in vivo* hearts from several species by perfusing large coronary arteries at constant coronary perfusion pressure, rather than constant flow. These investigators suggested that the coronary vasoconstrictor response to ACh may indirectly result from the production of local metabolites, secondary to ACh's negative inotropic effect; however, the pig was not studied. In several experiments, we deflated the balloon to unload the left ventricle and thus decrease myocardial oxygen demands, which should have altered the production of metabolites. We found no significant change in the vasoconstrictor response to ACh under these conditions. Therefore, we believe it is unlikely that alterations in myocardial metabolism from impaired left ventricular pressure development contributed significantly to the dramatic increases in coronary perfusion pressure that we observed in these piglet hearts in response to ACh.

Our data indicate that ACh can cause a weak coronary vasodilation at low doses (<1.0 nmol). Similarly, Corr *et al.* (32) found that ACh, at low concentrations (10^{-6} M), could produce relaxation of isolated rabbit epicardial coronary arteries, whereas at higher concentrations (>10^{-6} M) only vasoconstriction was observed. These results indicate that there may be more than one endothelium-derived factor or mechanism governing the effect of ACh on the coronary vasculature. Our data also suggest that the vasoconstrictor response to ACh was greater in hearts from older piglets. Additionally, the reduction in left ventricular PSP for these neonatal pig hearts was much greater than the negative inotropic effects produced by ACh in *in vivo* adult pig hearts (8). We speculate that these results may indicate that there are maturational changes in responses to ACh, with the coronary vasoconstrictor effect increasing and the negative inotropic effect decreasing with age. Additional experiments will be required to confirm this hypothesis.

In conclusion, our study demonstrates that ACh primarily produces dose-dependent coronary vasoconstriction and a negative inotropic effect in isolated, neonatal pig hearts perfused at constant coronary flow with an erythrocyte-enriched solution. There is, however, supporting evidence with our preparation that ACh has a modest vasodilatory effect when administered at low doses. Our data also suggest that a prostanoid may mediate in part ACh's coronary vasoconstriction and that an extracellular source of calcium participates in this vasoconstriction.

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