Ca\textsuperscript{2+}-independent inhibition of myocardial contraction by coronary effluent of hypoxic rat hearts

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\textsuperscript{1}Department of Cardiology, University of Wales College of Medicine, Cardiff CF4 4XN; and \textsuperscript{2}Guy's, King's and St Thomas' School of Medicine, King's College London, London SE5 9PJ, United Kingdom

Yang, Zhao-Kang, Nick J. Draper, and Ajay M. Shah.\textsuperscript{1} Ca\textsuperscript{2+}-independent inhibition of myocardial contraction by coronary effluent of hypoxic rat hearts. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H623–H632, 1999.—Endothelial cells release agents that influence cardiac contraction. We recently reported that cultured hypoxic endothelial cells release an unidentified factor(s) that inhibits myocardial contraction. In this study, we investigated the effects of coronary effluent of isolated hypoxic rat hearts on isolated rat ventricular myocyte contraction. Coronary effluent collected during brief moderate hypoxia significantly depressed myocyte twitch shortening and decreased diastolic length, with only minor reduction in intracellular Ca\textsuperscript{2+} transients. These effects were similar to those of hypoxic coronary microvascular endothelial cell superflusates and were reversed by reoxygenation of hearts. “Hypoxic” coronary effluent exerted essentially Ca\textsuperscript{2+}-independent effects on myofilament interaction in intact myocytes, as assessed by 1) peak Ca\textsuperscript{2+}-shortening relations, 2) phase-plane analysis of instantaneous Ca\textsuperscript{2+}-cell length relations, and 3) “steady-state” myofilament responses in tetanized, sarcomplasmic reticulum-disabled cells. Thus an unidentified substance(s) that inhibits myocyte shortening predominantly via effects on the myofilaments is reversibly released during acute moderate hypoxia of isolated hearts, presumably from coronary endothelial cells. Release of such an agent may be relevant to the cardiac contractile response to hypoxia.

endothelium; myofilament; ischemia; adaptation

In recent years, it has become increasingly clear that the importance of the endothelium is not restricted to the modulation of vascular tone and homeostasis. Several studies have established that both the coronary vascular and the endocardial endothelium play a paracrine role in regulating cardiac contractile function (for reviews, see Refs. 1, 17, 27). Cardiac endothelial cells release several diffusible agents, including nitric oxide, endothelin, prostanoids, and kinins, that modify cardiac myocyte function. Such paracrine effects of endothelial cells have been demonstrated both in vitro and in vivo (11), confirming their likely importance.

Besides these known agents, endothelial cells release other unidentified substances that have potent effects on myocardial contraction. Both the superfusates of pure cultures of endothelial cells and the coronary effluent of isolated buffer-perfused rat hearts are reported to alter the contraction of isolated cardiac myocytes and cardiac trabeculae (5, 6, 12, 15, 18, 19, 21). The stimuli that influence the release of these substances from endothelial cells remain poorly understood. Ramaciotti et al. (15) reported circumstantial evidence suggesting that coronary flow rate and ambient P\textsubscript{O}\textsubscript{2} were important regulatory factors. Indeed, endothelial cell function (e.g., the release of vasoactive mediators) is known to be highly sensitive to acute alterations in flow-induced shear stress as well as acute moderate hypoxia (reviewed in Ref. 25). Recently, we reported that cultured large-vessel endothelial cells superfused with moderately hypoxic buffer (P\textsubscript{O}\textsubscript{2} 40–50 mmHg) for 1–6 h released a stable, low-molecular-mass substance(s) that induced a potent, reversible inhibition of cardiac myocyte shortening not attributable to reduction in cytosolic Ca\textsuperscript{2+} transients (19). This inhibitory effect appeared to be independent of subcellular second messenger signaling pathways. Thus this substance markedly depressed the translocation of actin filaments over myosin molecules in an in vitro motility assay and reduced the rate of actin-activated cardiac myosin ATPase activity in solution (19). The inhibitory activity was not attributable to generally recognized cardioactive or vasoactive substances released by endothelial cells.

If a similar substance were released by coronary endothelial cells in the whole heart in response to hypoxia, this could have important implications for the cardiac adaptive response to hypoxia. In the present study, we therefore 1) investigated the effects of the coronary effluent of acutely hypoxic isolated hearts on cardiac myocyte contraction, 2) compared this with the effects of hypoxic cultured coronary microvascular endothelial cell (CMEC) superfusate, and 3) studied the subcellular mechanisms underlying the effects of hypoxic coronary effluent.

METHODS

All studies conformed with the United Kingdom Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986. For isolation of hearts, cardiac myocytes, or CMEC, adult Wistar rats of either sex weighing 250–350 g were terminally anesthetized with pentobarbital sodium (60 mg/kg ip). Hearts were rapidly excised and placed into ice-cold HEPES buffer of the following composition (in mM): 117 NaCl, 5.7 KCl, 4.4 NaHCO\textsubscript{3}, 1.2 NaH\textsubscript{2}PO\textsubscript{4}, 1.25 CaCl\textsubscript{2}, 1.7 MgCl\textsubscript{2}, 20 HEPES, and 10 glucose, pH 7.4 at 37°C.

Collection of coronary effluent from isolated perfused rat hearts. Isolated hearts were mounted on a nonrecirculating Langendorff apparatus and retrogradely perfused with HEPES buffer (37°C, gassed with 100% O\textsubscript{2}, P\textsubscript{O}\textsubscript{2} > 680 mmHg). Indomethacin (10 \textmu M) was included to inhibit cyclooxygenase, and acebutolol (1 \textmu M) was included to inhibit any...
β-adrenergic effects secondary to catecholamine release. A constant coronary flow that achieved a mean perfusion pressure of 70–80 mmHg was maintained. Hearts were paced at ~10% above intrinsic rate by a right atrial electrode at ~10% above threshold voltage. A water-filled latex balloon attached to a SensoNor 840 transducer was used to measure isovolumic left ventricular pressure. Left ventricular end-diastolic pressure was set at ~10 mmHg. Left ventricular and coronary perfusion pressures were recorded on a chart recorder. Hearts in which mean coronary perfusion pressure or left ventricular pressure varied by >5% or those that had significant arrhythmia during an equilibration period of 25 min were excluded from the study.

After equilibration, coronary effluent was collected during continued normoxic perfusion for at least 5 min. Perfusion was then switched to hypoxic HEPES buffer (gassed with N2 for > 30 min, PO2 ~35 mmHg) for 5 min, and after this period hearts were reoxygenated. Consecutive 5-min collections of coronary effluent were performed. Effluents were studied either fresh or after a period of storage at 4°C. There were no significant changes in ionic composition (Na+, K+, Mg2+, Cl−, and Ca2+), osmolality, or pH of the effluents compared with the perfusing buffer.

Endothelial cell isolation and culture. CMEC were isolated and cultured as described previously (13). Briefly, hearts mounted on a Langendorff apparatus were perfused at 37°C with buffer 1 of the following composition (in mM): 118 NaCl, 4.7 KCl, 1.2 NaH2PO4, 1.2 MgSO4, 25 NaHCO3, and 11 glucose, pH 7.4 (gassed with 95% O2-5% CO2). Epicardial mesothelial cells were devitalized with 70% (vol/vol) ethanol. Buffer 1, with the addition of 0.25 µM CaCl2 and 0.04% collagenase (type I, Sigma Chemical), was then perfused and recirculated for 30 min. Ventricles (excluding visible large vessels) were chopped into 15 ml of recirculating solution containing 200 mg of bovine serum albumin (fraction V, Sigma Chemical) and triturated gently at 37°C for 10 min. The suspension was filtered through nylon gauze and centrifuged (150 g, 3 min) to sediment myocytes, and the supernatant, with the addition of 100 mg of bovine serum albumin, 0.01% trypsin, and 50 µM CaCl2, was incubated at 37°C for 15 min. The CMEC pellet was obtained by centrifugation (1,000 g, 10 min), washed twice in buffer 1 with 250 and 500 µM CaCl2, respectively, and resuspended in 40 ml of prewarmed medium 199 (GIBCO) with 10% newborn calf serum, 10% fetal calf serum, 250 U/ml benzylpenicillin, 250 µg/ml streptomycin, 12.5 µg/ml amphotericin B, and 50 µg/ml gentamycin. Cell suspensions were plated in 75 cm² tissue culture flasks (37°C). After 1 h, unattached cells and debris were washed off. CMEC were cultured to confluence (5–7 days) in medium 199 (with added serum and antibiotics as above) and then subcultured in fresh flasks. CMEC were characterized as endothelial by their typical “cobblestone” morphology, uptake of fluorescently labeled acetylated low-density lipoprotein by >99% of cells, and negative staining for smooth muscle α-actin or characterized as microvascular by the rapid formation of capillary-like tubes on the basement membrane preparation Matrigel (8). Porcine aortic endothelial cells were cultured exactly as described previously (19). At passages 2–4, rat CMEC and porcine aortic endothelial cells were transferred to siliconized stirrer vessels with 3–4 ml of microcarrier beads and maintained on beads for ~1–2 wk until use.

Endothelial cell superfusion. Paired identical aliquots of confluent cells on microcarrier beads (1.5–2 ml; i.e., 5 × 106 cells) were washed with HEPES buffer and placed in cartridges with 0.8-µm filters (19). Cartridges were continuously superfused with HEPES buffer at 1 ml/min (37°C) for ≤6 h. Both cartridges were initially superfused with normoxic buffer (PO2 160 mmHg). After 1 h, the solution superfusing one cartridge was switched to hypoxic buffer (PO2 40–50 mmHg), whereas the other cartridge continued to be superfused with normoxic buffer. Matched single-pass superfusates of both cartridges were collected over consecutive 60-min intervals and stored at −70°C. The ionic composition (Na+, K+, Mg2+, Cl−, and Ca2+), osmolality, and pH of superfusates were not significantly altered during the period of study. Endothelial cell viability was also unaltered during either normoxic or hypoxic superfusion, as confirmed by trypan blue exclusion and the absence of lactate dehydrogenase in the superfusates.

Isolation of ventricular myocytes and assessment of myocyte function. Ventricular myocytes were isolated by Ca2+-free collagenase digestion and were loaded with the Ca2+-sensitive fluorescent probes fura-2 AM or indo-1 AM (6 and 10 µM, respectively) as described previously (19). At least 45 min were allowed for deesterification of the indicators. Cells were studied within 8 h of isolation. A drop of myocyte suspension was placed in a chamber on the stage of a Nikon Diaphot inverted fluorescence microscope and superfused with HEPES buffered or test solutions (PO2 160 mmHg) at 1 ml/min. Single adherent cells were studied according to previously established criteria, i.e., they were rod-shaped and free of membrane blebs or granulation, with <1 spontaneous contractile event per minute and a stable contraction pattern. Experiments were performed at room temperature (23°C) to minimize cell leakage of fluorescent probes (23). Myocytes were field stimulated at 0.5 Hz. Cell length was monitored by either a custom-designed photodiode array system or a video edge-detection system (Crescent Electronics). The amplitude of unloaded twitch contraction is reported as the percent decrease in diastolic length. Fura 2 fluorescence was excited at 340 and 380 nm by a rotor-mounted system, and emission was measured at 510 nm. The ratio of fluorescence after excitation at these wavelengths (340/380 ratio) was used as an index of intracellular Ca2+ concentration ([Ca2+]i). Indo-1 fluorescence was excited at 360 nm, and the 410/480 nm emission ratio was recorded as an index of intracellular Ca2+. No attempt was made to calibrate Ca2+-sensitive fluorescent indicators (23). Data files of 6–10 consecutive steady-state beats (fluorescence ratio and cell length) recorded at intervals were averaged for analyses.

To assess the “steady-state” relationship between cell shortening and intracellular Ca2+ in intact single myocytes, cells were first pretreated in the chamber for 10 min with thapsigargin (0.4 µM) to irreversibly inhibit sarcoplasmic reticulum Ca2+ uptake. Subsequent repetitive rapid electrical stimulation (10 Hz for 10–20 s) resulted in a reproducible, steady elevation of intracellular Ca2+ accompanied by a steady tetanic shortening of the cell for the period of stimulation (12, 20). This allowed assessment of the steady-state intracellular Ca2+ concentration ([Ca2+]i)-shortening relationship in a single cell preparation with intact sarcolemmal membranes and intact subcellular signaling pathways.

For bioassay studies, all endothelial superfusates and coronary effluents were carefully reequilibrated for temperature, PO2 (~160 mmHg), and pH before testing was performed on isolated cardiac myocytes. As far as possible, matched sets of effluents collected during normoxia, hypoxia, and reoxygenation of individual hearts (i.e., “normoxic,” “hypoxic,” and “posthypoxic” samples) were each tested on individual cardiac myocytes. The difference between the effect of normoxic and hypoxic effluents on an individual myocyte was taken as the net “hypoxic effect” (19). This experimental design was used...
to minimize variation in effects between different effluents and in the responses of individual cardiac myocytes. A similar protocol was used for testing the endothelial cell superfusates. Matched effluents and superfusates were thus identical in every respect except for the difference in PO2 during heart or endothelial cell perfusion.

Effect of enzymatic hydrolysis on hypoxic coronary effluents. To investigate the chemical nature of the substance(s) in coronary effluent that exerted biological activity on cardiac myocytes, effluents were subjected to attempted enzymatic hydrolysis. The effects of acid phosphatase, 3'-nucleotidase, 5'-nucleotidase, trypsin, sulfatase, glutamic-pyruvic transaminase, and glutaminase were studied. Samples of hypoxic coronary effluent were split into two identical aliquots (~10–20 ml each), and one of the enzymes listed was added to one aliquot (0.01 U/ml). Both aliquots were then incubated at 37°C for 30 min, heated at 90°C to denature the enzyme, and centrifuged to remove denatured products. The paired aliquots were then tested in random order on individual cardiac myocytes, and the effect of the enzyme-treated aliquot was expressed as a percentage of the effect obtained with the untreated aliquot.

Materials. Fura 2-AM and indo 1-AM were purchased from Calbiochem, medium 199 with L-glutamine was from Gibco, and all other chemicals and reagents were from Sigma Chemical.

Statistics. Data are given as means ± SE. Comparisons were made by an unpaired or paired Student’s t-test, as appropriate, on absolute values, and differences were considered significant if \( P < 0.05 \).

RESULTS

Effect of coronary effluent collected during hypoxia. On exposure to coronary effluents collected during hypoxia, reductions in isolated myocyte twitch shortening ranging from ~10 to 100% depression were observed. The majority of this variability was at the level of the effluents and not the myocytes, i.e., any individual effluent had broadly similar effects on several cardiac myocytes.

Figure 1 shows an example of an experiment in which exposure to hypoxic effluent resulted in almost total abolition of the twitch. This was associated with a marked reduction in cell diastolic (resting) length. The inhibition of myocyte shortening occurred rapidly (within <1 min) on exposure to the hypoxic effluent.

Fig. 1. Example of potent effect of hypoxic coronary effluent on isolated rat cardiac myocyte contraction. A: slow time-base recording of cell twitch shortening during exposure to normoxic (i), hypoxic (ii; heavy horizontal bar), and posthypoxic coronary effluents (iii) collected from the same heart. All effluents were reequilibrated for temperature, PO2 (~160 mmHg), and pH before they were tested on isolated cardiac myocytes. B: fast time-base recordings of cell shortening and associated intracellular Ca2+ transients during exposure to effluents indicated.
Subsequent exposure of the myocyte to coronary effluent collected during reoxygenation (posthypoxic effluent) resulted in a rapid recovery of cell shortening back to the control level, whereas diastolic cell length usually recovered more slowly. Figure 1B shows fast time-base traces of single twitches and their associated fluorescence ratio transients recorded from this myocyte during the exposures indicated in Fig. 1A. It is notable that the abolition of myocyte twitch shortening by hypoxic coronary effluent was not accompanied by a corresponding reduction in amplitude of the intracellular Ca\(^{2+}\) transient. Subsequent exposure to posthypoxic effluent also resulted in no significant change in the Ca\(^{2+}\) transient.

In experiments in which hypoxic coronary effluent had less potent depressant effects on cardiac myocyte contraction, the pattern of effect was nevertheless similar, i.e., a reduction in myocyte shortening and diastolic length associated with a minor or no reduction in amplitude of Ca\(^{2+}\) transient (e.g., Fig. 2). To investigate possible reasons for the variability in depressant effects of hypoxic coronary effluents, the influence of several factors relating to the isolated hearts was assessed. The coronary effluents were divided into those that induced \(\geq 40\%\) reduction in myocyte twitch shortening (25 hearts) and those that induced <40% reduction (17 hearts). This cutoff point was chosen on the basis of the magnitude of reduction in myocyte twitch shortening previously found with the superfusates of hypoxic endothelial cells (19). Table 1 shows that these groups did not differ significantly with respect to animal weight, heart weight, pacing rate, coronary flow, coronary perfusion pressure, or left ventricular systolic function before or during hypoxia. Furthermore, there was no correlation between any of these parameters and the level of myocyte depressant activity in individual coronary effluents (data not shown).

Pooled data indicating the effects on myocyte assays of hypoxic coronary effluents with depressant effects \(\geq 40\%\) (collected from 9 hearts) are shown in Fig. 3. Relative to the stable contraction pattern during superfusion with HEPES buffer (i.e., the “control”), normoxic coronary effluent did not significantly alter twitch shortening (101.6 \(\pm\) 11.4% of control, \(n = 12\)). The hypoxic effluent decreased myocyte twitch shortening to 27.8 \(\pm\) 4.8% of control (\(P < 0.0001, n = 31\)), whereas the fluorescence ratio transient was decreased to 88.4 \(\pm\) 2.1% of control (\(P < 0.05, n = 31\)). Hypoxic effluent also decreased cell diastolic length from 94.7 \(\pm\) 2.8 to 92.0 \(\pm\) 3.0 µm (\(P < 0.001\)). The posthypoxic effluent had no significant effect on twitch shortening relative to control (97.4 \(\pm\) 3.1% of control, \(n = 20\)).

The inhibitory activity of hypoxic coronary effluent was stable at \(-70^\circ\text{C}\) for several months and was present in molecular mass fractions of \(<500\) daltons after ultracentrifugation through Centricon MWCO 500 cutoff filters (data not shown), similar to the findings previously reported for cultured endothelial cell superfusates (19).

Effect of superfusates of hypoxic CMEC. Superfusates obtained from two batches of rat cultured CMEC were studied. Figure 4 shows an example of a potent effect of hypoxic CMEC superfusate on myocyte shortening. In this cell, addition of hypoxic superfusate resulted in a rapid total inhibition of shortening that was not accompanied by a corresponding reduction in amplitude of the intracellular Ca\(^{2+}\) transient. Myocyte diastolic length was also significantly decreased. Replacement of hypoxic superfusate by posthypoxic superfusate resulted in a rapid recovery of cell shortening and diastolic cell length. These effects were similar to those observed with hypoxic coronary effluent (Fig. 1) and those previously reported with pig aortic endothelial cells (19).

Pooled data showing the effects of hypoxic superfusates of cultured rat CMEC and cultured pig aortic endothelial cells (3 batches) on myocyte contraction are shown in Fig. 5. On average, hypoxic CMEC superfusates decreased cell twitch shortening by \(\sim 40\%\) (relative

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**Table 1. Comparison of coronary effluents with high and low cardiodepressant activity**

<table>
<thead>
<tr>
<th>Twitch Depression, %</th>
<th>n</th>
<th>Animal Weight, g</th>
<th>Heart Weight, g</th>
<th>Pacing Rate, s(^{-1})</th>
<th>Coronary Flow, ml/min</th>
<th>CPP, mmHg</th>
<th>LVPSP, mmHg</th>
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<tr>
<td>(\geq 40)</td>
<td>25</td>
<td>347 (\pm) 12.3</td>
<td>1.40 (\pm) 0.04</td>
<td>298 (\pm) 3.9</td>
<td>10.5 (\pm) 0.4</td>
<td>72.2 (\pm) 2.3</td>
<td>97.3 (\pm) 2.0</td>
</tr>
<tr>
<td>(&lt; 40)</td>
<td>17</td>
<td>362 (\pm) 16.1</td>
<td>1.43 (\pm) 0.05</td>
<td>301 (\pm) 3.5</td>
<td>11.3 (\pm) 0.4</td>
<td>75.9 (\pm) 1.9</td>
<td>97.2 (\pm) 2.7</td>
</tr>
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Values are means \(\pm\) SE; \(n\) = no. of rat hearts. CPP, coronary perfusion pressure; LVPSP, left ventricular peak systolic pressure.
to normoxic superfusates) with a reduction in the Ca\(^{2+}\) transient of \(~10\%\). Cell diastolic length was also significantly reduced. Essentially identical effects were observed with the superfusates of pig aortic endothelial cells.

Assessment of calcium-myofilament interaction. Myocyte contractile amplitude is closely influenced by the peak intracellular Ca\(^{2+}\) and the myofilament responsiveness to Ca\(^{2+}\). The relative contribution of these factors to altered contraction may be assessed from the relation-

![Graph](image)

Fig. 3. Percent changes (relative to control HEPES buffer) in cell twitch shortening (TS; left) and amplitude of Ca\(^{2+}\) transients (fluorescence ratio as % of control) on exposure to coronary effluents. Numbers in parentheses indicate number of myocytes studied. *P < 0.05; ***P < 0.0001 vs. control.

Fig. 4. Example of potent effect of hypoxic superfusate of rat cultured coronary microvascular endothelial cells (CMEC) on isolated rat cardiac myocyte contraction. A: slow time-base recording of cell twitch shortening during exposure to normoxic (i), hypoxic (ii; heavy horizontal bar) and posthypoxic CMEC superfusates (iii). All endothelial superfusates were carefully reequilibrated for temperature, PO\(_2\) (~160 mmHg), and pH before they were tested on isolated cardiac myocytes. B: fast time-base recordings of cell shortening and associated intracellular Ca\(^{2+}\) transients during exposure to superfusates indicated.
ship between peak twitch shortening and peak intracellular Ca$^{2+}$ (fluorescence ratio). Figure 6 (A and B) shows this relationship for the twitches recorded from three myocytes that, after a period of quiescence, were electrically stimulated (0.5 Hz). With the use of linear regression as a simple and convenient analysis, the sequential reductions in fluorescence transient and twitch shortening during the “negative staircases” were found to be highly correlated. In contrast, there was a very weak correlation between peak fluorescence ratio and peak twitch shortening for contractions recorded during exposure of myocytes to hypoxic coronary effluents (expressed as a percentage of the respective baseline values before addition of effluents) (Fig. 6C). In other words, the changes in cell shortening were, to a large extent, unrelated to alterations in intracellular Ca$^{2+}$.

Further analyses of the instantaneous relation during single twitch contractions between intracellular Ca$^{2+}$ and cell length (as an index of myofilament activation) were made using phase-plane plots, as described previously (22). Figure 7 shows typical plots of instantaneous myocyte length versus fluorescence ratio during twitch contractions of a cell exposed to potent hypoxic coronary effluent (Fig. 7A) and a cell exposed to hypoxic CMEC superfusate (Fig. 7B). The phase-plane loops proceed counterclockwise, with cell shortening denoted by the ascending limb and cell relengthening by the descending limb. During exposure to either hypoxic coronary effluent or hypoxic endothelial superfusate, 1) cell length was shorter throughout the rising phase of the Ca$^{2+}$ transient, resulting in an upward shift of this phase relative to the control or normoxic loop, and 2) subsequent dynamic shortening of the cell was minimal, resulting in a dramatic compression of the loop in the vertical direction. Thus the “resting” myofilament response was apparently augmented, but there was a marked insensitivity to the rise and fall of the Ca$^{2+}$ transient. This pattern was quite distinct from the effect of 2,3-butanedione monoxime (BDM; 1 mM), which reduces myofilament Ca$^{2+}$ sensitivity (2) (Fig. 7C). In this case, the whole loop was shifted down and right, and cell length was longer relative to control at all levels of Ca$^{2+}$. The effects of hypoxic effluent and superfusate were also different from those of an intervention such as pyrophosphate, which decreased both twitch shortening and the Ca$^{2+}$ transient (see below); here, the loop area was symmetrically reduced in vertical and horizontal directions, with
minor change in the initial and terminal phases of the loop (Fig. 7D). Replacement of the hypoxic effluent or superfusate by posthypoxic solutions resulted in a return of the loop configuration to the prehypoxic baseline (Fig. 7, A and B).

The steady-state relationship between intracellular Ca\(^{2+}\) and cell shortening was assessed in intact tetanized myocytes. Figure 8 is an example of the effect of 5 min of exposure to moderately potent hypoxic coronary effluent on myocyte tetanic shortening and Ca\(^{2+}\). No significant change in the peak tetanic fluorescence ratio was observed. However, the resting cell length was shorter in the presence of the hypoxic effluent, whereas the amplitude of tetanic cell shortening was reduced relative to control. This effect was rapidly reversible (not shown). In five myocytes exposed to this hypoxic coronary effluent, the amplitude of tetanic shortening was reduced by 46.2 ± 9.5% (P < 0.01) whereas the tetanic fluorescence ratio transient was unchanged (−0.1 ± 4.8%, P = not significant).

Agent(s) responsible for activity of hypoxic coronary effluent. It was previously reported that the activity of hypoxic coronary effluent...
hypoxic endothelial cell superfusate) was not accounted for by known cardioactive factors released by living cells, 2) probably involved a low-molecular-mass, stable factor acting directly on the cardiac myofilaments independent of known subcellular signaling pathways, and 3) was specific for cardiac myosin and did not affect smooth muscle myosin (19). In view of these features, we considered the possibility that the inhibitory activity may involve a nucleotide or nucleoside analog or product that specifically inhibited cardiac but not smooth muscle myosin or that some intermediate product of high-energy metabolic pathways in the endothelial cell may be responsible. Accordingly, we surveyed the effects of several pharmacological agents on myocyte twitch shortening and Ca\(^{2+}\) transients (Table 2). However, none of these compounds reproduced the effects of the hypoxic coronary effluents or endothelial superfusates. A negative inotropic effect associated with a reduction in the intracellular Ca\(^{2+}\) transient was observed with four compounds. The changes in twitch shortening and fluorescence ratio with these compounds were as follows: adenosine 2'-monophosphate, -17.6 and -12.5%; adenosine 3'-monophosphate, -24.3 and -16.7%; adenosine 5'-O-(3-thiotriphosphate), -28.2 and -15.3%; and sodium pyrophosphate, -69.7 and -48.6%, respectively.

Attempted hydrolysis of hypoxic coronary effluent failed to significantly diminish the effect of effluent on myocyte function. After incubation with acid phosphatase, 3'-nucleotidase, 5'-nucleotidase, trypsin, sulfatase, transaminase, or glutaminase, the activities of hypoxic effluent were 127.2, 113.5, 129.9, 84.8, 102.5, 98.9, and 128.4%, respectively, of the matching untreated effluent (n = 2 for each).

**DISCUSSION**

The main finding of the present study is that isolated rat hearts respond rapidly and reversibly to acute moderate hypoxia by releasing an unidentified stable substance that inhibits isolated cardiac myocyte shortening and reduces diastolic cell length independent of changes in intracellular Ca\(^{2+}\). These effects are similar to those of the superfusate of hypoxic rat CMEC in culture (present study) and to those previously reported for hypoxic large vessel endothelial cells in culture (19). The action of hypoxic coronary effluent appears to result predominantly from a Ca\(^{2+}\)-independent modification of myofilament function as assessed both during twitch and tetanic contraction.

The present study significantly extends the previous data obtained in cultured large-vessel endothelial cells (19) in that it 1) establishes that the release during hypoxia of substances that inhibit myocyte contraction is not simply a cultured cell phenomenon but is applicable to the whole heart; 2) demonstrates that in the intact heart, this response can be evoked by just 5 min of moderate hypoxia and can also be rapidly “switched off” within 5 min of reoxygenation; and 3) suggests that the cardiodepressant factor(s) in coronary effluent of hypoxic rat hearts may derive from coronary endothelial cells in situ. The latter would be consistent with previous reports suggesting the presence of endothelial-derived cardioactive factors in the coronary effluent of isolated perfused rat hearts (5, 12, 15). However, the results of the present study do not exclude the possibility that other cell types within the heart may also play a role. The rapidity of release of the cardioactive factor is consistent with previous studies showing that acute moderate luminal hypoxia (PO\(_2\) 30–60 mmHg in buffer-perfused systems) is an effective stimulus for rapid changes in several aspects of endothelial function (25), including the release of vasodilators such as nitric oxide and prostanoids in isolated arteries and the intact circulation (9, 14). Because endothelial cells have a low “metabolic” sensitivity to hypoxia, with no change in high-energy phosphate content or cell viability for several hours even during severe hypoxia (PO\(_2\) <10 torr) (7), these rapid responses to moderate hypoxia may represent an undefined hypoxia “sensing” process unrelated to changes in energy production (14).

The chemical identity of the substance released in response to hypoxia is currently unknown. The activity was not attributable to any changes in measured ionic composition, osmolality, pH, or PO\(_2\) of the coronary effluent. We have previously reported (19) that the biological actions of a similar or identical substance released by cultured large vessel endothelial cells are not attributable to known cardioactive factors released by these cells (e.g., nitric oxide, adenosine, prostanoids, endothelin-1) and are found in low-molecular-mass fractions (relative molecular weight <500). Activity of the factor was maintained for several hours at room temperature and for several weeks at –70°C and was not significantly diminished by heating at 90°C. In the present study, we also investigated the possibility that the inhibitory activity may involve a nucleotide or nucleoside analog (or product) that specifically inhibited myofilament function or that some other intermediate product of high-energy metabolic pathways in the endothelial cell was involved. However, a survey of several potential candidate compounds failed to iden-
tify any that reproduced the typical actions of the factor released during hypoxia (Table 2). A significant negative inotropic effect of pyrophosphate (1 mM) was observed, but this was associated with a concomitant reduction in the Ca\(^{2+}\) transient. A number of the other compounds tested [adenosine 5'-O-(3-thiotriphosphate), adenosine 2'-monophosphate, and adenosine 3'-monophosphate] exerted small effects on myocyte shortening at high (\(\geq\)100 \(\mu\)M) doses, but these were also attributable to changes in the Ca\(^{2+}\) transient. We also found that activity was not significantly reduced by attempted enzymatic hydrolysis.

Inhibition of cardiac myocyte contraction by the endothelial factor without corresponding reduction in the intracellular Ca\(^{2+}\) transient suggests that this effect occurred predominantly via an interaction with the myofilaments, rather than any excitation-contraction coupling process capable of modulating cytosolic Ca\(^{2+}\) levels. In keeping with this, there was a poor correlation between changes in peak fluorescence ratio and alterations in twitch shortening on exposure to hypoxic coronary effluent, in contrast to the good correlation between these parameters during negative staircases (Fig. 6). Analysis of the instantaneous relationship between intracellular Ca\(^{2+}\) and cell length during electrically stimulated twitch contractions indicated that there was a marked insensitivity of cell length to changes in intracellular Ca\(^{2+}\) (Fig. 7). This was manifested as a loss of hysteresis in the "phase-plane loop." This effect was quite different from that observed with BDM, which reduces myofilament Ca\(^{2+}\) sensitivity (2). In the case of BDM, the hysteresis in the phase-plane loop was to a large extent maintained, but the position of the loop was shifted right and down, i.e., toward reduced myofilament responsiveness to Ca\(^{2+}\) (22). A further point of note was the reduction in cell length induced by the endothelial factor, even at low (diastolic) Ca\(^{2+}\) levels. The data obtained in experiments in which myocytes were electrically tetanized after inhibition of the sarcoplasmic reticulum Ca\(^{2+}\) ATPase by thapsigargin were consistent with these findings. Myocyte cell length was reduced by the endothelial factor, whereas the amplitude of cell shortening on steady elevation of intracellular Ca\(^{2+}\) was significantly depressed (Fig. 8). The underlying molecular mechanism(s) responsible for these changes remains to be defined. One possibility that we have suggested is that, in the presence of the endothelial factor, cross bridges are able to attach and generate force but that their cycling rate is reduced (19). This could occur, for example, because of an inhibition of the transition of cross bridges out of high force states, somewhat analogous to the latch-state cycling of smooth muscle in which force is maintained while cross bridges turn over more slowly (3). Such a mechanism would be, to the best of our knowledge, unique for any paracrine agent released by living cells.

There was a significant variability in the magnitude of effect of the hypoxic coronary effluent on isolated cardiac myocyte shortening, which was largely due to variability of effluents rather than of myocyte responses. This variability could not be accounted for by differences in baseline contractile function or coronary perfusion of the hearts or by the level of contractile depression during hypoxia (Table 1). All the animals used for these studies were similar in age, weight, source, and strain. Furthermore, great care was taken to ensure that conditions such as temperature, pH, ionic composition, and PO\(_2\) were precisely controlled. A more likely possibility that may account for the observed variability in response is that the endothelial factor could be released abluminally and that what was detected in the coronary effluent might represent "spillover" into the coronary circulation. Such a mechanism is known to exist for other endothelial cell-derived factors, e.g., endothelin-1 (26). It is also feasible that the endothelial factor might be degraded in the coronary circulation and that this process may be variable. The precise reasons for the variability remain to be defined; in preliminary studies, we have found that the proportion of heart effluents that exert significant reduction in myocyte twitch shortening can be increased to a maximum of \(~70%\) by recirculating the effluent collected during hypoxia and concomitantly reducing coronary flow rate to \(~66\)% of the baseline level during hypoxia (unpublished data).

Because the present study was performed using isolated buffer-perfused hearts, extrapolation of the findings to the situation in vivo involves many untested assumptions. Nevertheless, our results could be of relevance to certain cardiac pathological conditions. The level of isolated heart hypoxia studied (PO\(_2\) \(\sim\)35 Torr) was within a pathophysiologically relevant range that might occur during partial reduction in coronary blood flow (10). The release of a factor that inhibits myocyte shortening in response to hypoxia could serve to reduce energy turnover and myocardial O\(_2\) consumption. Such a mechanism may therefore function to facilitate the maintenance of myocardial oxygen supply-demand balance during hypoxia or partial coronary flow restriction. Indeed, it is recognized that oxygen supply-demand balance during acute hypoxia or a temporary partial reduction in coronary flow may be facilitated by a stable decrease in oxygen demand associated with a proportional depression of contractile force (4, 16). Restoration of oxygen supply or coronary flow usually leads to prompt recovery of function. The mechanism(s) responsible for this adaptive downregulation of oxygen demand remains unclear, but a sustained decrease in high-energy phosphates or phosphorylation potential is not thought to be responsible. Our findings raise the possibility that coronary microvascular endothelial cells, sited at the interface between vascular O\(_2\) supply and the O\(_2\)-utilizing tissue, may "sense" hypoxia and trigger adaptive responses such as the release of the contraction-inhibiting substance described in the present study. Alternatively, it is also feasible that such a factor could "protect" against the deleterious effects of Ca\(^{2+}\) overload during prolonged hypoxia and/or immediately on reoxygenation. By causing the cardiac myofilaments to become relatively insensitive to rises in intracellular Ca\(^{2+}\), such a factor could minimize cellular hypercontracture at reoxyge-
The effects of Ca2
stance exerts Ca2
in intact cardiac myocytes, it appears that this sub-
subcellular mechanisms of action of this agent in
hearts subjected to ischemia, although the effects and
release of a cardiodepressant mediator by isolated
hypoxia, the effects on cardiac myocytes in situ would
factor is "turned off" rapidly on cessation of brief
normal twitch contraction.) Whereas release of the
myocyte shortening is still lower than that during
ing length of externally unloaded cells, the extent of
ation. (Although the "hypoxic factor" reduces the rest-
ing length of externally unloaded cells, the extent of
myocyte shortening is still lower than that during
normal twitch contraction.) Whereas release of the
factor is "turned off" rapidly on cessation of brief
hypoxia, the effects on cardiac myocytes in situ would
In conclusion, we have shown that the reoxygenated
ory response of coronary endothelial cells to hyp-
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