Soluble Substances Released From Postischemic Reperfused Rat Hearts Reduce Calcium Transient and Contractility by Blocking the L-Type Calcium Channel

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OBJECTIVES	This study was designed to investigate the effects of cardiodepressant substances released from postischemic myocardial tissue on myocardial calcium-regulating pathways.
BACKGROUND	We have recently reported that new cardiodepressant substances are released from isolated hearts during reperfusion after myocardial ischemia.
METHODS	After 10 min of global ischemia, isolated rat hearts were reperfused, and the coronary effluent was collected for 30 s. We tested the effects of the postischemic coronary effluent on cell contraction, Ca^{2+} transients and Ca^{2+} currents of isolated rat cardiomyocytes by applying fluorescence microscopy and the whole-cell, voltage-clamp technique. Changes in intracellular phosphorylation mechanisms were studied by measuring tissue concentrations of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), as well as activities of cAMP-dependent protein kinase (cAMP-dPK) and protein kinase C (PKC).
RESULTS	The postischemic coronary effluent, diluted with experimental buffer, caused a concentration-dependent reduction of cell shortening and Ca^{2+} transient in the field-stimulated isolated cardiomyocytes of rats, as well as a reduction in peak L-type Ca^{2+} current in voltage-clamped cardiomyocytes. The current reduction resulted from reduced maximal conductance—not from changes in voltage- and time-dependent gating of the L-type Ca^{2+} channel. The postischemic coronary effluent modified neither the tissue concentrations of cAMP or cGMP nor the activities of cAMP-dPK and PKC. However, the effluent completely eliminated the activation of glycogen phosphorylase after beta-adrenergic stimulation.
CONCLUSIONS	Negative inotropic substances released from isolated postischemic hearts reduce Ca^{2+} transient and cell contraction through cAMP-independent and cGMP-independent blockage of L-type Ca^{2+} channels. (J Am Coll Cardiol 2001;37:668–75) © 2001 by the American College of Cardiology

We have recently reported that previously unknown and still unidentified negative inotropic substances (NIS) are released from the postischemic myocardium [1,2]. The present study was performed to analyze the mechanism of the negative inotropic effects mediated by NIS. We used isolated rat cardiomyocytes as an in vitro bioassay system in which the effects of postischemic coronary effluent from isolated rat hearts were analyzed. In isolated ventricular myocytes, contractility can be quantified by the extent and rate of unloaded shortening [3]. Because contractile activation is controlled by the Ca^{2+} transient (i.e., by changes in cytosolic Ca^{2+} concentration $[Ca^{2+}]_c$ [4]), the effects of the postischemic coronary effluent on cell shortening and Ca^{2+} transient of isolated cardiomyocytes were studied together. The Ca^{2+} transient is primarily based on Ca^{2+} release from the sarcoplasmic reticulum triggered by Ca^{2+} influx through sarcolemmal L-type Ca^{2+} channels [3–6]. We therefore conducted a separate group of experiments with whole-cell, voltage-clamp experiments to test the effect of NIS on the L-type Ca^{2+} channel current, I_{Ca}. As phosphorylation mechanisms are involved in L-type Ca^{2+} channel regulation [7,8], we conducted a further series of experiments to address the question of whether NIS modulate Ca^{2+} channel activity by changes in tissue concentrations of cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP).

METHODS

Collection of coronary effluent from isolated rat hearts after myocardial ischemia. Isolated rat hearts were perfused in the noncirculating mode at a constant flow (10 ml/min) with a modified Krebs-Henseleit buffer (KHB), as described elsewhere [1,2]. The hearts were subjected to 10 min of global stop-flow ischemia followed by reperfusion. The postischemic coronary effluent was then collected immediately at the onset of reperfusion, over a period of 30 s. The coronary effluent from 20 postischemic

Abbreviations and Acronyms

BSA	= bovine serum albumin
cAMP	= cyclic adenosine monophosphate
cAMP-d PK	= cAMP-dependent protein kinase
cGMP	= cyclic guanosine monophosphate
DMSO	= dimethyl sulfoxide
KHB	= Krebs-Henseleit buffer
NIS	= negative inotropic substances
pA	= picoampere
pF	= picofarad
PKC	= protein kinase C
pS	= picosiemeus
rfu	= relative fluorescence units
SR	= sarcoplasmic reticulum

hearts was pooled, and aliquots of the pooled solution were stored at $-70°C$ before testing. Postischemic pooled coronary effluent was diluted with experimental buffer. In addition, the coronary effluent of five control hearts, with no previous ischemia, was collected and pooled by identical techniques.

Isolation of ventricular myocytes. Adult isolated rat hearts were perfused with oxygenated Ca^{2+}-free KHB (37°C, pH 7.4) containing (in mmol/liter) 110 NaCl, 2.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 HEPES and 11 glucose. After 3 min, the hearts were perfused with KHB containing 30 μmol/liter of Ca^{2+} and collagenase type II (355 U/ml). After 30 min of collagenase digestion, the hearts were minced and incubated for another 15 min in the same solution. The following washing steps increased $(Ca^{2+})_o$ incrementally, in steps of 200, 500 and 1000 μmol/liter. The cells were next layered over a 4% bovine serum albumin (BSA) gradient and centrifuged for 1 min at 19 g; the resulting pellet was then resuspended in the experimental buffer (in mmol/liter: 117 NaCl, 2.8 KCl, 0.6 $MgCl_2$, 1.2 KH_2PO_4, 1.2 $CaCl_2$, 20 glucose and 10 HEPES (pH 7.3). Typically, about 2×10^6 cells per rat heart were obtained, 95% of which showed the typical rod-shaped morphology with no blebs or granulation.

The cells were plated on four-well chamber glass slides (Nunc, Naperville, Illinois), which had been coated with 10 μg/ml of laminin. After an attachment period of 30 min, the buffer was exchanged for a staining solution containing 0.1% dimethyl sulfoxide (DMSO), 0.025% Pluronic F-127, 0.2% BSA and 5 μmol/liter of Fluo 3-AM. The cells were incubated at room temperature for 45 min on an orbital shaker, with oscillation of 40 rpm. After incubation, the loading solution was replaced with fresh buffer, and the incubation continued for 30 min.

Measurement of Ca^{2+} transients and cell shortening. The cells were superfused continuously with experimental buffer at a flow rate of 2 ml/min and kept at room temperature to minimize the cell leakage of fluorescent probes (9). They were field-stimulated by a custom-built stimulator through bipolar silver-chloride electrodes at a frequency of 1 Hz for a duration of 5 ms. After a 2-min

equilibration period with 1-Hz stimulation, the coronary effluent was superfused.

Cell shortening and Ca^{2+}-dependent changes in Fluo-3 fluorescence of single-ventricular myocytes were simultaneously measured by use of an Odyssey XL confocal laser scan microscope (Noran Instruments, Middleton, Wisconsin), using an argon ion laser with 6 mW of excitation at 488 nm. Emitted light was long-pass–filtered (>515 nm) and collected at a frame rate of 120 images/s. Data were stored on the hard disk of an Indy workstation (Silicon Graphics, Mountain View, California). At an interval of every 8 ms, Ca^{2+} transients and cell length were determined using a custom-written macro function for Object-Image (10). Under control conditions, the rat ventricular myocytes (n = 36) shortened during stimulation by $7.3 \pm 0.4\%$ (mean $\pm$ SEM). Changes in Ca^{2+} transients are calculated as peak systolic relative fluorescence units (rfu) minus diastolic rfu without the calibration effort, owing to the uncertain subcellular compartmentalization of the probes (9). At baseline, Fluo-3 fluorescence increased from diastolic 23.1 ± 1.9 rfu to peak systolic 59.1 ± 5.4 rfu.

Electrophysiology. Rat cardiomyocytes were isolated as described previously (11,12) and superfused with saline solution (37°C) containing (in mmol/liter) 150 NaCl, 5.4 KCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 20 glucose and 5 HEPES, with adjustment by NaOH to pH 7.4. For whole-cell, patch-clamp experiments, borosilicate glass capillaries of 2 mm in diameter were pulled to the tips of $\sim$2 μm in diameter (1.5 to 2 MΩ resistance). Currents were recorded with a RK300 input amplifier (Biologic, Echiroll, France), filtered at 2 kHz and sampled at 5 kHz. Superimposed K^+ currents were reduced by Cs^+ electrode solution, as described elsewhere (12). In the whole-cell mode of the voltage clamp, 100- or 140-ms pulses to 0 mV were applied at 1 Hz. The pulses started from a holding potential of -45 or -50 mV to inactivate the Na^+ channel currents (3,12). Experiments with seal resistance <2 GΩ were discarded. Membrane capacitance was measured by a train of 10 pulses between -45 and -50 mV. The average membrane capacitance was 121 ± 1.4 picofarad (pF) (58 cells). No data correction took place for membrane leakage. The I_{Ca} was expressed by current densities (picoampere [pA]/pF after division by cell capacitance) to remove the variability of I_{Ca} with cell size.

Effects of the coronary effluent on myocardial tissue concentrations of cAMP and cGMP: activities of cAMP-dPK, PKC and glycogen phosphorylase. Isolated rat hearts were separately perfused at a constant flow (10 ml/min), which was followed by serial perfusion, as described elsewhere (1,2). At the onset of serial perfusion, the coronary effluent of a donor heart (heart no. 1) was reoxygenated and transported to a serially perfused second heart (heart no. 2). Serial perfusion of the two hearts began: 1) after 30 min of separate perfusion at a constant flow or 2) after 20 min of separate perfusion and 10 subsequent min of global ischemia of heart no. 1.
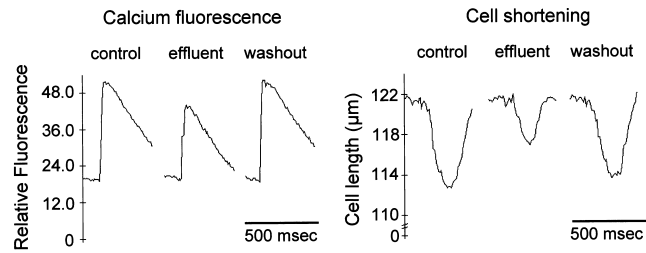
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Calcium fluorescence

Cell shortening

Figure 1. Fast time-base recording of changes in Fluo-3 fluorescence, expressed as relative fluorescence units (rfu) and cell length (μm) of a single field-stimulated rat cardiomyocyte under basal conditions (control), during superfusion with postischemic coronary effluent (dilution 1:4) and during superfusion with fresh experimental buffer (washout). Effluent = postischemic coronary effluent.

Heart no. 2 was freeze-clamped 30 s after the onset of serial perfusion for biochemical examination. Tissue levels of cAMP were assayed according to Gilman (13), after purification by column chromatography, as described elsewhere (14). Cyclic GMP contents were analyzed by radioimmunoassay, according to the technique of Harper et al. (15). Cardiac cAMP-dPK activity was measured in the particulate tissue fraction according to a modified method of Murray et al. (16). Cyclic AMP-dPK activity was expressed as the activity ratio of malantide phosphorylation in the absence and presence of 2.8 μmol/liter of cAMP. Activity of PKC was determined in the cytosolic and particulate fractions, as described elsewhere (17,18), using the PKC kit (BIOTRAK, Braunschweig, Germany). The activity of glycogen phosphorylase b to a transformation (expressed as percent total activity) was estimated according to the method of England (19). Protein was measured as described elsewhere (20), with use of ovalbumin as the standard agent.

Ethics. The investigation conforms to the "Position of the American Heart Association on Research Animal Use," adopted by the Association in November 1984.

Statistical analysis. The results are expressed as the mean value $\pm$ SEM. For comparison of different groups, Kruskal-Wallis analysis of variance was performed, followed by the one-sided Mann-Whitney U test at $p < 0.05$. For comparison of different values in the same group, the one-sided Wilcoxon matched pairs test was performed at $p < 0.05$. Adjustments of the post hoc test for multiple comparisons (more than two groups) were carried out using a sequentially rejective test procedure (Bonferroni-Holm).

Materials. Unless otherwise stated, substances were purchased from Sigma Chemicals (Deisenhofen, Germany). Collagenase type II was purchased from Worthington (Freehold, New Jersey); BayK8644 from Bayer Pharmaceutical (Leverkusen, Germany); and (^{32}P)-γ-ATP, (^{3}H)-cAMP, the (^{125}J)-cGMP assay system and the PKC kit BIOTRAK from Amersham-Buchler. BayK8644 and nifedipine were prepared in DMSO, and aliquots were stored at -20°C. Further dilutions were made in experimental buffer.
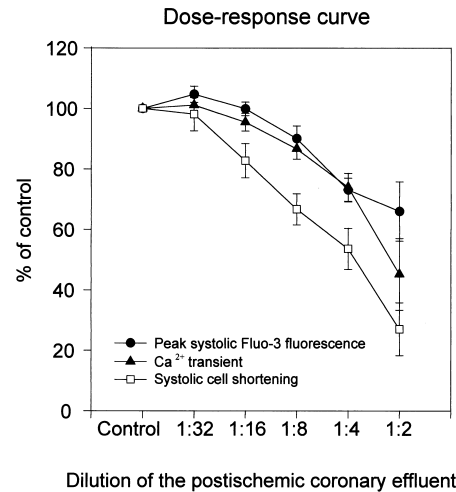
Dose-response curve

Dilution of the postischemic coronary effluent

Figure 2. Effects of pooled postischemic coronary effluent at different dilutions on systolic cell shortening, Ca^{2+} transient (peak systolic rfu − diastolic rfu) and peak systolic Fluo-3 fluorescence of isolated field-stimulated rat cardiomyocytes. The effects were calculated as percent changes compared with the values under basal conditions (control). The data are presented as the mean value $\pm$ SEM for six different myocytes incubated with the indicated dilutions of postischemic effluent. Each cardiomyocyte was used for only a single study employing one of five different dilutions.

RESULTS

Reduction of cell shortening and Ca^{2+} transient. Figure 1 shows the original fast-time base recording of cell shortening and (Ca^{2+})$_c$ fluorescence of a field-stimulated cardiomyocyte under control conditions and during superfusion of postischemic coronary effluent, diluted to 1:4. The effluent reduced the Ca^{2+} transient and systolic cell shortening by 25% and 38%, respectively. The effect of the effluent was reversible during superfusion with fresh experimental buffer (washout).

Figure 2 shows the dependence of systolic cell shortening, Ca^{2+} transient and peak systolic Fluo-3 fluorescence on the concentration of postischemic coronary effluent. At all dilutions tested, a decrease in Ca^{2+} transient and systolic cell shortening reached steady-state within 1 min and was reversible within 1 min on washout. The effects of the effluent were evaluated once stable steady-state conditions were reached. Increasing concentrations of postischemic coronary effluent depressed cell contraction to a greater extent than they did Ca^{2+} transient. Diastolic Fluo-3 fluorescence did not change significantly. Superfusion of undiluted nonischemic coronary effluent modified neither cell contraction nor Ca^{2+} transient (n = 6; data not shown).

Signaling pathways. DEPRESSION OF THE CA^{2+} CHANNEL CURRENT, I$_{CA}$. Figure 3A shows the net membrane current of a rat ventricular myocyte produced by clamp-step depolarization from -45 to 0 mV for 100 ms. The K$^+$ outward currents were largely blocked by cell dialysis with Cs$^+$ ions, and Na$^+$ inward current was inactivated by starting the clamp step from the holding potential of -45 mV. The resulting inward current can accordingly be attributed to
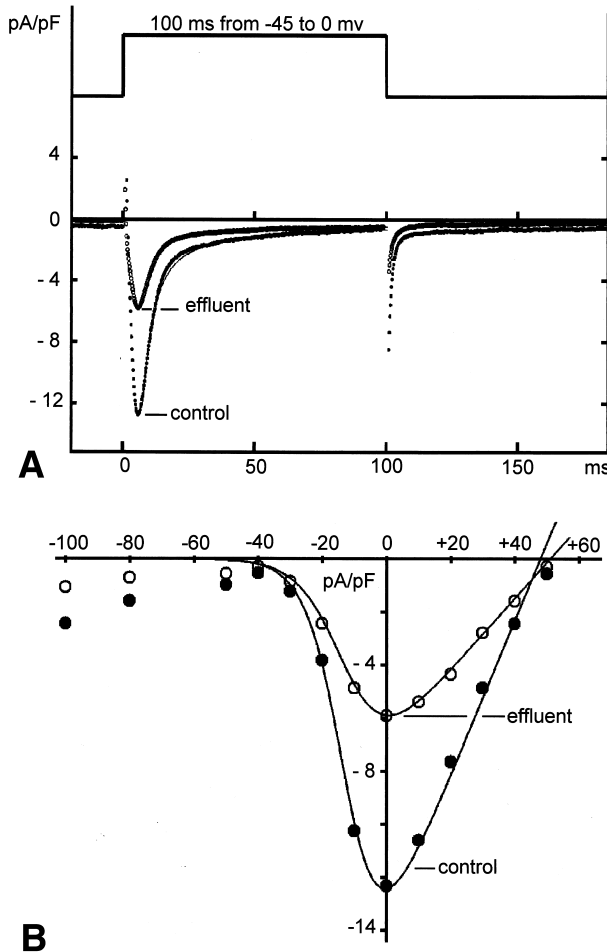
A

B

Figure 3. Reduction of whole-cell I_{Ca} by the postischemic coronary effluent. **A,** I_{Ca} recorded from a rat ventricular myocyte before (control; **solid circles)** and 1 min after adding 1:4 diluted postischemic effluent to the superfusate **(open circles).** The time-dependent decay of I_{Ca} was matched to a double-exponential function **(thin solid line**; time constants = 4 and 20 ms). **B,** Plot of peak I_{Ca} as a function of the test-step potential (iv curve) before (control; **filled circles)** and after adding 1:4 diluted postischemic effluent to the superfusate **(open circles).** The holding potential was -45 mV. Between -40 and 50 mV, voltage dependence of peak I_{Ca} is matched, as calculated from the Boltzman formula. Effluent = postischemic coronary effluent (dilution 1:4).

L-type I_{Ca} (11). In Figure 3A, I_{Ca} peaks within 3 ms to -12.4 pA/pF and decays along a double-exponential time course (thin-fitting lines in Fig. 3A [11]). The fast exponential demonstrated a time constant of 4 ms; its amplitude contributed to the peak current by 74%. The slow exponential had a time constant of 20 ms and contributed with an amplitude of 26%. Superfusion of pooled postischemic coronary effluent diluted by 1:4 reduced the peak I_{Ca} from -12.4 to -5.3 pA/pF (Fig. 3A). The effect became steady within ~1 min. No change took place in the time to peak current. It was possible to fit the decay of the effluent-modified I_{Ca} to the same time constants of 4 and 20 ms and to a very similar ratio of the amplitudes (78% and 22%, respectively). These last results indicate that the kinetics of the decay were insensitive to the effluent. Accordingly, we conclude that the reduction of peak I_{Ca} by the postischemic
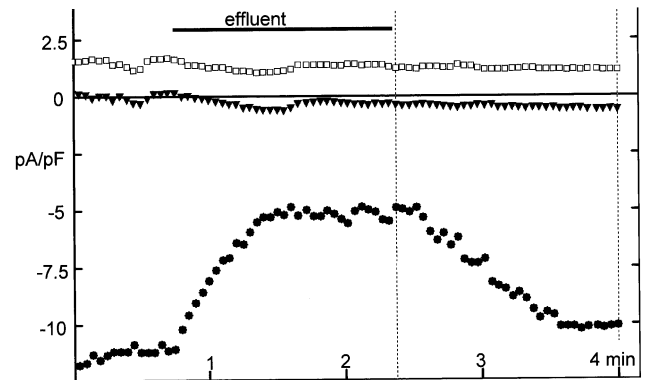

Figure 4. Time course of the changes in peak I_{Ca} **(filled circles)**, the holding current at -50 mV **(open squares)** and the late current (current measured at the end of the 100-ms pulse to 0 mV; **filled triangles)** during superfusion with postischemic coronary effluent (dilution 1:4) and during washout.

effluent is unlikely to be caused by changes in the activation or inactivation variables. Nonischemic coronary effluent collected from isolated control hearts did not modify the I_{Ca} of the isolated cardiomyocytes (data not shown).

The voltage dependence of peak I_{Ca} (Fig. 3B) is typical for currents through Ca^{2+} channels of the L-type. The fit of the corresponding current-voltage (iv) curves (according to the Boltzman formula [3]) yielded the following gating variables for the control currents: -12 mV for the potential of half-maximal activation, -6.2 mV for the slope factor and 48 mV for the reversal potential. Figure 3B indicates that the effluent did not change these steady-state gating variables. In the presence of the postischemic coronary effluent diluted to 1:4, these variables yielded: -12 mV, -6.8 mV and 51 mV, respectively. However, it reduced the whole-cell Ca^{2+} conductance from 644 to 289 picosiemens [pS]/pF, a decrease of 55%, as evaluated from the slope of the iv curve.

The effect of the postischemic effluent on the Ca^{2+} inward current was reversible on superfusion with experimental buffer. Figure 4 shows the time course for the development of this effect on peak I_{Ca} during superfusion and washout of the postischemic effluent. The postischemic coronary effluent diluted by 1:4 suppressed peak I_{Ca} within 1 min to a new steady-state value. During superfusion with fresh experimental buffer (washout), the effect of the postischemic coronary effluent on peak I_{Ca} was reversible within 1 min.

On a statistical basis, the pooled postischemic coronary effluent significantly reduced the peak I_{Ca} in the cells tested. In cells with complete iv curves, the potential of half-maximal activation, the slope factor and the reversal potential were not significantly changed. The reduction of peak I_{Ca} reached steady-state within 1 min. The time dependence of the decay in I_{Ca} did not change significantly (p > 0.05). The paired comparison indicated a reduction of peak I_{Ca} by 54%, from -11.8 ± 0.7 to -5.4 ± 0.4 pA/pF (n = 9, p < 0.05) when the postischemic coronary effluent was

Table 1. Tissue Levels of Cyclic Adenosine Monophosphate and Cyclic Guanosine Monophosphate and Activities of Cyclic Adenosine Monophosphate-Dependent Protein Kinase and of Protein Kinase C in Serially Perfused Heart No. 2: Effect of Nonischemic and Postischemic Coronary Effluent of Heart No. 1

					PKC	
						Particulate Fraction
	cAMP	**cGMP**		**Cytosolic fraction**		
			cAMP-dPK	**(pmol/min per**	**(pmol/min per**	
Condition of Serial Perfusion	**(pmol/mg protein)**		**(−cAMP/+cAMP)***	**mg protein)**	**mg protein)**	**(%)†**
Nonischemic coronary effluent	2.89 ± 0.23	0.21 ± 0.02	0.21 ± 0.01	940 ± 46	149 ± 22	13.7 ± 2.1
	(n = 11)	(n = 7)	(n = 4)	(n = 6)	(n = 6)	(n = 6)
Postischemic coronary effluent	3.63 ± 0.47	0.25 ± 0.02	—	986 ± 83	203 ± 49	17.1 ± 3.4
	(n = 5)	(n = 6)		(n = 5)	(n = 5)	(n = 5)
Isoproterenol, 5 μmol/liter‡						
Nonischemic coronary effluent	6.19 ± 0.95§	0.21 ± 0.05	0.29 ± 0.02§	—	—	—
	(n = 5)	(n = 5)	(n = 5)			
Postischemic coronary effluent	6.37 ± 1.04§‖	0.23 ± 0.05	0.30 ± 0.03§	—	—	—
	(n = 5)	(n = 5)	(n = 5)			

*Given as the quotient calculated from the activities obtained without adding and after adding cAMP. †Percent of total PKC activity. ‡Isoproterenol was infused to heart no. 2 at the onset of serial perfusion. §p < 0.05 vs. serial perfusion with nonischemic effluent in the absence of isoproterenol. ‖p < 0.05 vs. serial perfusion with postischemic effluent in the absence of isoproterenol. Data presented as the mean value ± SEM; number of hearts is given in parentheses.

cAMP = cyclic adenosine monophosphate; cGMP = cyclic guanosine monophosphate; cAMP-dPK = cAMP-dependent protein kinase; PKC = protein kinase C.

diluted to 1:4, and by 80%, from −12.1 ± 0.6 to −2.4 ± 0.3 pA/pF (n = 9, p < 0.05) when the dilution was 1:2.

Maximal Ca^{2+} conductance was significantly reduced, by 56 ± 2.7% (p < 0.05), during exposure to postischemic coronary effluent (dilution 1:4). The slope conductance between −60 and 100 mV was not significantly influenced (31 ± 3 pS/pF before and 25 ± 2 pS/pF after addition of postischemic coronary effluent) (p > 0.05). Likewise, the holding current at −50 mV was not significantly changed by the postischemic coronary effluent (Fig. 4). In only five of nine cells, the inward currents between −60 and −100 mV tended to become more negative (as shown in Fig. 3B). On a statistical basis, this effect was not significant. We attributed the minimal reduction of inward currents to a more complete block of K^+ currents by Cs^+ when cell dialysis increased the cytosolic Cs^+ concentration with time.

Reduction of I_{Ca} by the postischemic coronary effluent does not depend on prestimulation by isoproterenol or BayK8644. We investigated whether the reduction of peak I_{Ca} by the postischemic coronary effluent (dilution 1:4) depended on the status of the Ca^{2+} channel, as it may be influenced by cAMP-dependent phosphorylation or by Ca^{2+} channel openers. Application of 30 nmol/liter of isoproterenol (n = 6) increased peak I_{Ca} from −12.6 ± 1 to −19.5 ± 1.2 pA/pF, and addition of the postischemic coronary effluent reduced prestimulated peak I_{Ca} within 1 min to −10.5 ± 1.2 pA/pF (i.e., by 54%) (p < 0.05). Similarly, addition of 2 μmol/liter of 8-Br-cAMP (n = 4) increased peak I_{Ca} from −11.5 ± 1.1 to −20.8 ± 1.7 pA/pF, and the postischemic coronary effluent reduced I_{Ca} to −10.3 ± 1.6 pA/pF. Superfusion of 0.5 μmol/liter of BayK8644 (n = 5) increased peak I_{Ca} from −11.8 ± 0.8 to −43.7 ± 1.8 pA/pF, and the postischemic coronary effluent reduced prestimulated peak I_{Ca} to −14.5 ± 1.1 pA/pF (p < 0.05). When peak I_{Ca} was reduced by superfusion of 0.1 μmol/liter of nifedipine to −4 ± 0.6 pA/pF, addition of the postischemic coronary effluent induced a further reduction

to −1.7 ± 0.3 pA/pF (n = 3). In summary, our results indicate that the reducing effect of the postischemic coronary effluent on the amplitude of peak I_{Ca} does not depend on the status of the channel, as it is modified by cAMP-dependent phosphorylation or by dihydropyridines.

Changes in tissue levels of cAMP and cGMP and activities of cAMP-dPK and PKC. To establish whether the observed effects of the postischemic coronary effluent are modulated by an intracellular phosphorylation/dephosphorylation mechanism of the L-type Ca^{2+} channel, we conducted serial perfusions of two isolated hearts. We measured the tissue levels of cAMP and cGMP, as well as the activity of cAMP-dPK in serially perfused heart no. 2. As summarized in Table 1, the basal levels of cAMP and cGMP in heart no. 2 were not significantly influenced by the postischemic coronary effluent of heart no. 1, compared with nonischemic effluent. The tissue level of cAMP in heart no. 2 was elevated by isoproterenol stimulation (5 nmol/liter). We then investigated whether the postischemic coronary effluent of heart no. 1 had an antiadrenergic effect in heart no. 2 through cAMP-mediated signaling, compared with nonischemic effluent. As shown in Table 1, there were no significant differences between the two groups in generation of cAMP or in the cAMP-dPK activity ratio. The myocardial tissue level of cGMP did not increase significantly in the presence of isoproterenol in the two groups.

To preclude the possibility that signaling cascades other than beta-adrenoceptor signaling may be involved in the effects of the postischemic coronary effluent, we also measured the distribution of PKC activity in the cytosolic and particulate fractions of the heart. As shown in Table 1, PKC activity in heart no. 2 was not influenced by the postischemic coronary effluent (Table 1).

Activity of glycogen phosphorylase. We studied activation of glycogen phosphorylase because this enzyme is regulated by phosphorylase kinase, which in turn is activated

by either cAMP-dPK or an increase in $(Ca^{2+})_c$ (21–23). The glycogen phosphorylase b to a transformation, measured in the presence of AMP, increased in heart no. 2 from $7.5 \pm 1.3\%$ (controls, n = 4) to $49.9 \pm 3.0\%$ during intracoronary infusion of 5 nmol/liter of isoproterenol (n = 5) (p < 0.05). When serial perfusion was performed after 10 min of global ischemia of the first heart (n = 5), the activation of glycogen phosphorylase during exposure to isoproterenol (5 nmol/liter) was reduced to $9.9 \pm 2.5\%$ (p < 0.05 vs. values during infusion of isoproterenol without preceding ischemia of heart no. 1).

DISCUSSION

Negative inotropic substances released from isolated postischemic hearts. The present study investigated the mechanism by which NIS cause negative inotropic effects in the isolated, field-stimulated ventricular myocytes of rats. Our results indicate that the postischemic coronary effluent contains NIS that decrease contractility (cell shortening) by depression of Ca^{2+} transient, rather than by Ca^{2+} desensitization. Furthermore, our results reveal that the substance(s) reduce Ca^{2+} influx through L-type Ca^{2+} channels. Accordingly, we postulate that this Ca^{2+} channel blockage is the initial event in the signaling cascade.

The chemical structure of NIS has remained unknown until now. As evidenced by data that we have recently reported, the substance(s) involved here are stable, heat-resistant molecules that, most likely, are not proteins (1). We have excluded the possibility that ionic imbalances, lactic acid or pH shifts in the pooled coronary effluent of the postischemic hearts contributed to its effects on contraction and Ca^{2+} transient of the isolated cardiomyocytes. The substances(s) unidentified thus far are small molecules: the pooled effluent was dialyzed against water (1:1000) by means of a diaphragm with a pore size of 0.5 kd. The remaining dialyzed solution—containing molecules >0.5 kd—once lyophilized and dissolved in an appropriate experimental buffer, did not influence cell shortening or Ca^{2+} fluorescence of isolated cardiomyocytes. In contrast, when the postischemic coronary effluent was filtered through a 0.5-kd filter (YCO5 Amicon membrane, Millipore, Eschborn, Germany), its effects on the calcium transients and cell shortening were comparable to those of untreated postischemic coronary effluent (data not shown).

Endogenous negative inotropic factors have been identified by other investigators using different experimental procedures. Splanchnic hypoperfusion induces production of a myocardial depressant factor in the splanchnic region (24). Myocardial depressant factor is a low molecular weight peptide that decreases myocardial contractile force. In addition, a cardiodepressant factor has been isolated by column chromatography from the plasma of dogs after hypovolemic-traumatic shock (25). Further purification yielded a hydrophilic peptide. Finally, several studies have shown that endocardial and coronary vascular endothelium

modulate myocardial function (26–28). Endothelial cells have been shown to release unidentified mediators that upregulate or downregulate myocardial contractility (28). A recent study has also disclosed the presence—in the superfusate of cultivated vascular and endocardial endothelial cells—of an unidentified low molecular weight factor that reduces myocardial contraction, predominantly by reducing the myofilament response to calcium (29). In contrast, we have recently provided evidence that the negative inotropic mediators released from postischemic hearts are not derived from endothelial cells (2).

Reduction of the L-type Ca^{2+} current. The voltage-clamp analysis described in the present study indicates that NIS reduce the L-type Ca^{2+} current I_{Ca} and intracellular systolic Ca^{2+} concentration, with the consequence of negative inotropy. In addition, a reduced intracellular Ca^{2+} concentration causes metabolic effects, such as a decrease in activation of glycogen phosphorylase. In contrast to our data, Yang et al. (30) recently reported that the coronary effluent of isolated hypoxic rat hearts contains unidentified substances that depress contraction of isolated ventricular myocytes, with only a minor reduction in intracellular Ca^{2+} transient. Furthermore, hypoxic endothelial cells produce diffusible factors that inhibit the myosin cross-bridge function (31). The apparent discrepancies between these reports and our data are most likely due to different experimental protocols. The cited reports describe superfusate of hypoxic endothelial cells or coronary effluent from hypoxic hearts; in our study, the isolated hearts were subjected to 10 min of stop-flow ischemia, followed by reperfusion. We therefore hypothesize that the substances, unidentified until now, that are released during reperfusion and after ischemia are different from those released during hypoxia.

The L-type Ca^{2+} channel activity is known to be up-regulated by cAMP and downregulated by cGMP-dependent phosphorylation (7,8). The present results indicate that the suppressing effect of NIS on I_{Ca} is not modified by prestimulating the Ca^{2+} channel activity through cAMP-dependent phosphorylation (with either isoproterenol or 8-Br-cAMP). Hence, modulation of the cAMP-dPK pathway by NIS seems unlikely. This conclusion is in accordance with our findings that the postischemic coronary effluent did not modify cAMP tissue concentration or cAMP-dPK activity in serially perfused isolated hearts. Similarly, signaling through cGMP or PKC is unlikely, because cGMP tissue levels and PKC activity were not modulated by the effluent. Because the postischemic coronary effluent blocked the I_{Ca} independently of the aforementioned mechanisms that initiate Ca^{2+} channel activation, it appears unlikely that dephosphorylation of subunits of the L-type Ca^{2+} channel is involved. We therefore postulate that NIS interact with the Ca^{2+} channel more directly (i.e., by plugging the pore or by binding to the channel protein) (32). Binding of NIS to the dihydropyridine binding site of the channel protein is unlikely, because suppression of I_{Ca} by the effluent was not modified when the

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cells were pretreated with the dihydropyridines BayK8644 or nifedipine. The mode of interaction between NIS and the Ca^{2+} channel remains to be elucidated by forthcoming single-channel studies.

Potential pathophysiologic role of the negative inotropic substances. In an attempt toward teleologic interpretation of our findings, it may be considered whether the observed reduction in Ca^{2+} influx, Ca^{2+} transient and cell shortening could serve as an endogenous protection mechanism. In terms of cardiac energy balance, the decrease in Ca^{2+} transient and in contractility may reflect a salutary effect that allows enhanced metabolic recovery of the cardiomyocytes before full contractility is restored. Furthermore, the question arises whether the effects of NIS interfere with the phenomenon of "stunning" (33,34). The potential underlying mechanisms of this postischemic contractile abnormality are the release of free radicals, cellular Ca^{2+} overload and decreased sensitivity of the contractile filaments to calcium (reviewed in [35]). Overload of Ca^{2+}, in concert with oxygen free radicals, may induce activation of Ca^{2+}-dependent protease activity and consequent troponin I proteolysis (36). Reduction in the L-type Ca^{2+} current by NIS may therefore represent a compensatory mechanism of the myocardium, in the form of counteracting Ca^{2+} overload during reperfusion. However, the apparent rapid reversibility of the negative inotropic effect during washout may suggest that NIS play only a minor role in changes of contractility of the postischemic myocardium. In contrast, it may be possible that NIS are continuously released into the microenvironment and retained by the postischemic myocardial tissue. With its use of spillover from the mediators into the coronary effluent, our experimental study may well have failed to accurately ascertain the tissue concentrations of NIS in the postischemic ventricle. These concentrations, indeed, may be much higher (and may decrease over a greater span of time) than those in the coronary effluent.

Conclusions. The present data confirm the release of NIS during reperfusion of isolated hearts after ischemia. Our findings indicate that these substance(s) or these mediator(s) of yet unknown chemical structure reduce the L-type Ca^{2+} current. Presumably, the Ca^{2+} channel blockage is the first step in a chain of cellular events leading to suppressed cytosolic Ca^{2+} transient and, in turn, to reduced contractions. Because the putative L-type Ca^{2+} channel antagonistic effect of NIS was not mediated by changes in cAMP or cGMP levels, nor by changes in cAMP-dPK and PKC activities, we postulate that there is a phosphorylation-independent interaction between NIS and the Ca^{2+} channel.

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