Nicorandil Reduces the Basal Level of Cytosolic Free Calcium in Single Guinea Pig Ventricular Myocytes

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ABSTRACT. Using fluorescent Ca^{2+} indicator fura-2 and whole-cell patch-clamp techniques, we examined the effect of 2-nicotinamidoethyl nitrate (nicorandil) on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and electrical properties in single guinea pig ventricular myocytes. Nicorandil (10 nM ~ 1 mM) reduced the resting level $[Ca^{2+}]_i$ monitored as fura-2 fluorescence ratio in a concentration-dependent manner. Dibutylryl guanosine 3':5'-cyclic monophosphate (cyclic GMP), a membrane permeable cyclic GMP analogue, mimicked the nicorandil action. Neither application of caffeine (10 mM) nor deprivation of extracellular Na^+ ions could prevent the nicorandil action on $[Ca^{2+}]_i$. In contrast, the nicorandil effect was virtually blocked by sodium orthovanadate (40 μ M), a Ca^{2+} pumping ATPase inhibitor. During electrophysiological experiments, nicorandil shortened action potential durations (205 ± 80 ms to 153 ± 76 ms) by increasing a glibenclamide-sensitive outward K^+ conductance. However, the drug produced little hyperpolarization (~ 2 mV) because the resting potential of ventricular myocytes was close to the K^+ equilibrium potential. The involvement of voltage-dependent Ca-channel current and Na-Ca exchanger was considered to be minimal under physiological conditions. It is thus concluded that nicorandil decreases basal $[Ca^{2+}]_i$ via cyclic GMP-mediated activation of the plasma membrane Ca^{2+} pump in guinea pig ventricular myocytes.

Among K^+ -channel opening agents, 2-nicotinamidoethyl nitrate (nicorandil), a nitrate vasodilator, was first noted to increase the potassium conductance in both vascular smooth and myocardium (13, 22, 24, 38). Nicorandil was more recently shown to open cardiac adenosine 5'-triphosphate (ATP)-sensitive K^+ channels in guinea pig ventricular cells (18) as were other K^+ channel openers (cromakalim: 11, 33, pinacidil: 2; for review, 8). These findings led us to consider that nicorandil may modulate $[Ca^{2+}]_i$ through a voltage-gated membrane current system, such as the Na-Ca exchanger (27, 28) or Ca^{2+} channels.

In addition, nicorandil shares some properties with other nitrates which increase intracellular guanosine 3':5'-cyclic monophosphate (cyclic GMP) level by activating a soluble guanylate cyclase in both smooth and cardiac muscles (9, 10, 13, 19). Using guinea pig heart cells, we recently demonstrated that human recombinant atrial natriuretic peptide (α hANP), which is known to increase intracellular cyclic GMP through the activation of a membrane-bound guanylate cyclase (ANP-receptor; 6), reduced the resting $[Ca^{2+}]_i$ level by a cyclic GMP-dependent activation of plasma mem-

brane Ca^{2+} pumps (34). Based on these previous studies, it was hypothesized that nicorandil might also decrease basal $[Ca^{2+}]_i$ in a cyclic GMP-dependent manner.

To clarify the action of nicorandil on $[Ca^{2+}]_i$ and its underlying mechanism(s), we examined the nicorandil effect on $[Ca^{2+}]_i$ and ionic currents in isolated guinea pig ventricular myocytes. Nicorandil was found to reduce the resting level of $[Ca^{2+}]_i$ through the cyclic GMP-mediated activation of the plasma membrane Ca^{2+} pump. A preliminary account of this study has appeared in abstract form (21).

METHODS

Cell preparation and $[Ca^{2+}]_i$ estimation by fura-2. Single ventricular myocytes were isolated enzymatically from adult guinea pigs of either sex (350–500 g) using collagenase (Yakult, YK101, Tokyo, Japan) as described previously (20). To load with fura-2, the myocytes (2×10^4) were suspended in 2 ml of Tyrode solution containing 10 μ M fura-2/AM (Molecular Probes Inc., Junction City, OR, U.S.A.) and 5% bovine serum at 37°C for ~ 30 min. A sample of the cells was then washed 2 times with bovine serum-free Tyrode solution by a low speed centrifugation and resuspended in 2 ml Tyrode

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solution at 4°C for >2 hours to facilitate the hydrolysis of fura-2/AM before experiments. The myocytes were then transferred to a small recording chamber (0.5 ml in volume) that had a glass coverslip bottom placed on the stage of an inverted microscope (Nikon, type TMD, Japan). Continuous superfusion with the Tyrode solution (~25°C, 2 ml/min) was started after the cells had settled down firmly on the bottom of chamber.

The content of the Tyrode solution was (in mM): NaCl 136.5, NaH₂PO₄ 0.3, KCl 5.4, CaCl₂ 1.8, MgCl₂ 0.5, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES); pH was adjusted to 7.4 with NaOH. The Na-free bathing solution contained (in mM): N-methyl D-glucamine 149, MgCl₂ 5, HEPES 5 and pH 7.4 by HCl. All reagents were purchased from Sigma Chemical Co. (St. Louis, U.S.A.).

The method for [Ca²⁺]_i estimation was the same as reported previously (34, 36). Briefly, fluorescence images at excitation wavelengths (bandwidth 11 nm) of 340, 360, and 380 nm were collected from single fura-2/AM-loaded myocytes via a silicon intensified target (SIT) camera (C2400-08H, Hamamatsu Photonics Ltd., Hamamatsu, Japan). They were fed to an image processing computer (Argus 100/Ca; Hamamatsu Photonics, Japan), digitized at 512 × 512 pixels every 1–3 s, and the fluorescence intensity ratio was calculated in a given pixel at each excitation wavelength after background subtraction. Excitation light was automatically filtered and altered using a motor-driven exchange wheel controlled by the computer.

The mean ratio was collected from a square region of interest that contained a single myocyte, and was plotted as a function of time (usually for 10 min). Signal ratio of 340 nm/380 nm was found to be more sensitive to small [Ca²⁺]_i changes than that of 340 nm/360 nm, because the 380 nm signal altered reversely in comparison with the 340 nm signal while the fluorescence at 360 nm stayed constant. We therefore employed the mean value of 340 nm/380 nm instead of 340 nm/360 nm to estimate [Ca²⁺]_i in a given myocyte. The fluorescent ratio varied considerably among experiments. Since it is difficult to evaluate the absolute value of [Ca²⁺]_i using fura-2/AM (1, 4, 17; for review, 26), relative changes in [Ca²⁺]_i were expressed as percentage of basal level, which was measured in Tyrode solution (1.8 mM Ca²⁺) unless otherwise described. In the early stage of study, however, *in vitro* calibration was made to calculate the absolute [Ca²⁺]_i in calibration solutions containing (in mM): KCl 147, NaCl 2, MgCl₂ 1, HEPES 10, ethyleneglycol-bis (β-aminoethylether) N, N'-tetraacetic acid (EGTA) 1, fura-2 (Molecular Probes Inc.) 0.002, and appropriate amount of CaCl₂ (pCa 9~5) (34, 36).

Patch clamp experiments. A List EPC-7 (Darmstadt, Germany) was used to clamp either whole-cell voltage or current of isolated cells (16). Pyrex glass pipettes were filled with a solution composed of (in mM): K-aspartate 100, KCl 20, MgCl₂ 3, K₂ adenosine 5'-triphosphate (ATP) 3, Na₂ creatine phosphate 5, Na₂ guanosine 5'-triphosphate 0.2, EGTA 5, HEPES 5 and pH 7.4 by KOH. Pipettes had a resistance of 2~3 MΩ when filled with this solution. To minimize fast Na- and T-

type Ca-channel currents during the voltage-clamp experiment, the membrane potential was held at -40 mV and usually 1–2 μM tetrodotoxin (TTX; Sankyo Inc., Tokyo, Japan) was added to the bathing Tyrode solution. As described in the results, either glibenclamide or CoCl₂ was applied when necessary to minimize ATP-sensitive K-channel or voltage-dependent Ca-channel currents, respectively.

Membrane currents were elicited by applying 300-ms voltage-pulses from the holding potential of -40 mV to various test potentials at 0.2 Hz. Current and voltage data were stored on a video tape via a pulse code modulation system (PCM501; Sony, Tokyo, Japan) for later analysis by a computer (PC98XL; NEC, Tokyo, Japan) with a sampling frequency of 2 KHz. The experiments were carried out at ~33°C. Nicorandil was a generous gift of Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). Numeral data are expressed by mean ± standard deviations with the number of observations in parentheses (n). Statistical differences were evaluated by Student's paired *t*-test and were considered significant at *P* < 0.05.

RESULTS

Nicorandil reversibly reduces [Ca²⁺]_i in a dose-dependent manner. Figure 1 shows representative fluorescence ratio images of fura-2-loaded myocytes before and after the application of 0.1 mM nicorandil. At every pixel in the display, fluorescence ratio (340 nm/380 nm) was divided into 15 color codes corresponding to ratio values between 1.43 and 2.68 in this experiment. It is evident that the exposure to nicorandil produced a reversible decrease in ratio values in each myocyte, corresponding to a reduction in [Ca²⁺]_i. Mean ratio changes of these myocytes for 10 min are depicted in Fig. 2A. Mean ratios were decreased from 2.05 to 1.86 in the myocyte to the right of figure (upper trace in Fig. 2A), and from 1.92 to 1.73 in the myocyte to the left, equivalent to reduction of 9.3% and 9.9% (lower trace), respectively.

Although we did not systematically convert the signal ratio to absolute [Ca²⁺]_i, in preliminary experiments, Ca²⁺ concentrations in resting cardiomyocytes were calculated using an *in vitro* calibration (34, 36; see also Methods). *K_d* values for fura-2 thus determined were usually higher (200~240 nM) than its originally reported *K_d* value (140 nM, 15). In quiescent rod-shaped myocytes, resting [Ca²⁺]_i averaged 122 ± 35 nM (n = 22). Since these [Ca²⁺]_i levels were approximately half of the *K_d* in our *in vitro* calibration, which was largely shifted to the right, a decrease in the signal ratio induced by nicorandil (e.g., 5~10%) indicated a substantial [Ca²⁺]_i reduction (~20 nM).

In 68 quiescent rod-shaped myocytes, fluorescence signal ratios were measured as illustrated in Figs. 1 and 2A, and percentage changes in ratio before and after application of nicorandil are summarized as a function of

drug concentrations in Figure 2B. Nicorandil produced a concentration-dependent reduction in $[Ca^{2+}]_i$, causing half maximum inhibition at $\sim 1 \mu M$ and saturating at $\sim 0.1 mM$.

Since nicorandil belongs to the nitrate family, which is generally known to activate a soluble guanylate cyclase, we tested whether a drug-induced increase in intracellular cyclic GMP levels was causally related to the $[Ca^{2+}]_i$ reduction. As we have previously reported (34), a similar range of $[Ca^{2+}]_i$ reduction was observed in cell treated with dibutyryl cyclic GMP (1 mM), a membrane-permeable cyclic GMP analogue.

Sarcolemmal Ca^{2+} pump plays a central role in the nicorandil-dependent regulation of $[Ca^{2+}]_i$. Since the result so far obtained suggested that nicorandil reduced basal $[Ca^{2+}]_i$ presumably through an increase in intracellular cyclic GMP levels as a cytosolic second messenger, we next addressed the question of which subcellular mechanism played an important role in the nicorandil-

dependent $[Ca^{2+}]_i$ regulation. Figure 3A depicts a typical experiment using caffeine. Caffeine was used to deplete an intracellular non-mitochondrial Ca^{2+} store (presumably, sarcoplasmic reticulum; SR) and to examine the contribution of the SR. Caffeine acts by opening SR Ca^{2+} release channels (23). Even after Ca^{2+} depletion by 10 mM caffeine, nicorandil significantly decreased basal $[Ca^{2+}]_i$. Similar reductions were consistently obtained in 12 myocytes, and mean percent reductions were $6.0 \pm 1.5\%$ (6) with 0.1 mM and $8.2 \pm 2.5\%$ (6) with 1 mM nicorandil ($P < 0.05$). It is thus unlikely that an accelerated Ca -uptake into the SR is involved in the nicorandil-dependent reduction of $[Ca^{2+}]_i$.

The cardiac Na-Ca exchange is electrogenic and in part determines basal $[Ca^{2+}]_i$ because it exchanges 3 Na^+ for 1 Ca^{2+} . Its reversal potential (E_{rev}) is positive to the resting potential under physiological conditions, and therefore contributes a basal outward movement of Ca^{2+} ions. Deprivation of extracellular Na^+ ions inhib-

Fig. 1. Nicorandil reversibly reduces the fura-2 signal.

Four consecutive fluorescence ratio images from two myocytes in color mode: panel A: control, B: 1.5 min after exposure of the cells to 0.1 mM nicorandil, C: 2.5 min after the extracellular application of nicorandil and D: 1.5 min after wash-out of the compound. Scales to the right of images indicate 15 grades corresponding the ratio from 1.43 to 2.68.

its the sarcolemmal Na-Ca exchanger (27, 28). Figure 3B illustrates an experiment to test the involvement of the Na-Ca exchanger. In this series of experiments, external Na^+ ions were removed and replaced with N-methyl D-glucamine. In 10 myocytes, nicorandil consistently produced a significant reduction in $[\text{Ca}^{2+}]_i$ and mean percentage reductions were $4.2 \pm 1.4\%$ (5) at 0.1 mM and $6.3 \pm 2.0\%$ at 1 mM (5) ($P < 0.05$). The Na-Ca exchanger does not primarily work to reduce $[\text{Ca}^{2+}]_i$ in this experimental condition.

As shown in Figure 3C, however, nicorandil effect was largely inhibited by prior exposure of cells to 40 μM sodium ortho-vanadate (Na_3VO_4). Mean percent reductions in $[\text{Ca}^{2+}]_i$ were $1.0 \pm 1.5\%$ (10) with 0.1 mM and $0.5 \pm 1.5\%$ (10) with 1 mM nicorandil (N.S.; $P > 0.05$). Vanadate (a pentacoordinate, trygonal bipyramid stereo analogue of phosphate) is known to block the

plasma membrane Ca^{2+} pump at a very low concentration (2–3 μM) by non-competitive inhibition of the binding of ATP to the high affinity site of the pump in a variety of cells (5, 29). Thus, nicorandil appeared to decrease $[\text{Ca}^{2+}]_i$ through cyclic GMP-dependent activation of the plasma membrane Ca-pump.

Changes in electrical membrane properties are not primarily involved in the nicorandil-induced $[\text{Ca}^{2+}]_i$ reduction. The external Na-free condition somehow lessened nicorandil effects on $[\text{Ca}^{2+}]_i$. In the next series of experiments, we therefore examined the possibility that nicorandil, acting as a K^+ channel opener, potentiates the Na-Ca exchanger by producing the hyperpolarization (which in turn increases the driving potential for the exchanger and expels more Ca^{2+} from the cytosol). In order to evaluate the nicorandil effect on electrical membrane properties of guinea pig ventricular myo-

Fig. 2. Time- and dose-dependence of nicorandil effect.

A: Temporal changes of mean fluorescence ratios in Fig. 1.

B: Fluorescence ratios were measured in the steady state in the absence and presence of various concentrations of extracellular nicorandil. Percent change in the ratio was measured in a given myocyte, and summarized as the function of nicorandil concentrations. Vertical bars indicate standard deviations and numbers in parentheses the number of cells examined.

Table I. EFFECTS OF NICORANDIL (0.1 mM) ON ELECTRICAL PARAMETERS.

	control	nicorandil
RMP (mV)	-75 ± 5.2	-77 ± 4.5 (5)
APA (mV)	132 ± 6.5	128 ± 7.8 (5)
APD ₅₀ (ms)	181 ± 68	$138 \pm 60^*$ (5)
APD ₁₀₀ (ms)	205 ± 80	$153 \pm 76^*$ (5)

note: RMP, resting membrane potentials, APA, action potential amplitudes, APD₅₀ and APD₁₀₀, action potential durations at 50% and 100% repolarization. *, $P < 0.01$.

Fig. 3. Effects of caffeine, extracellular Na-free, and sodium ortho-vanadate on the nicorandil action.

Nicorandil (0.1 mM) was extracellularly administered for the period indicated by horizontal bars at 10 min after initial treatment of 10 mM caffeine (A), 10 min after removal of external Na^+ ions (B), or 5 min exposure to 40 μM sodium orthovanadate (Na_3VO_4 ; C). Ordinates indicate percent changes of fluorescence ratio.

cytes, we conducted whole-cell patch-clamp experiments with relatively wide-tipped pipettes containing 3 mM ATP and 5 mM creatine phosphate. Table 1 summarizes pooled data from 5 current-clamped myocytes. Nicorandil reversibly shortened action potential durations (APD), but caused virtually no hyperpolarization. The drug-induced APD shortening was completely reversed by 1 μ M glibenclamide, an anti-diabetic sulfonylurea and blocker of I_{KATP} (35, 37).

Figure 4 shows a typical voltage-clamp experiment to further characterize the underlying mechanism for the nicorandil effect on membrane potentials. The myocyte was bathed with 5.4 mM K^+ Tyrode solution and was voltage-clamped to various potentials every 5 s from the holding potential of -40 mV, before and after application of 0.1 mM nicorandil. Original current traces at

Fig. 4. Nicorandil activates glibenclamide-sensitive K-currents. **A:** Membrane currents were elicited by various pulse potentials after blocking Na- and Ca-currents by 2 μ M TTX and 3 mM $CoCl_2$. Steady state current-voltage (I-V) relations were obtained from the original current traces as shown in upper panel. Lower panel shows three I-V relations: filled circles; control, open circles; those recorded 10 min after application of 0.1 mM nicorandil, and filled triangles; those 15 min after the subsequent addition of 1 μ M glibenclamide. **B:** Nicorandil-sensitive currents are shown as difference I-V relationships in the absence (open squares) and presence of glibenclamide (filled squares).

-90 mV and $+20$ mV are shown in the upper inset. Steady-state current-voltage relationships (Fig. 4A) indicated that nicorandil produced both outward and inward currents (indicated by open and closed circles), which were completely reversed by 1 μ M glibenclamide (filled triangles).

Figure 4B depicts the nicorandil-sensitive current (open squares) and its inhibition by glibenclamide (filled squares). The E_{rev} of drug-induced current was -75 mV in this experiment, and averaged -78 ± 4 mV (4). At 20 mM external K^+ ($[K^+]_o$), where 14.6 mM NaCl in the bathing Tyrode solution was replaced with equimolar KCl, the reversal potential was -45 ± 6 mV (3). Thus, the E_{rev} at both 5.4 mM and 20 mM $[K^+]_o$

Fig. 5. Voltage-gated L-type Ca-current is not affected by nicorandil.

A and B: L-type Ca-current was recorded by applying 50 mV depolarizing pulse from -40 mV to $+10$ mV before (**A**) and after extracellular application of 0.1 mM nicorandil (**B**). The myocyte was superfused with glibenclamide (1 μ M) 10 min prior to nicorandil application. Dotted lines indicate zero current levels and arrowheads peak inward Ca-currents. Cell capacitance: 75 pF.

C: Peak inward current-voltage relationships obtained from the same experiment in panels A and B. Test pulses between -40 mV and $+40$ mV were applied at 0.2 Hz in the absence (open circles) or presence (filled circles) of nicorandil and after wash-out (open triangles). Peak inward currents were measured as the difference between peak inward and steady state (end of 300 ms pulses) and were plotted as a function of test potentials.

was close to K^+ equilibrium potentials (E_K : -82 mV and -49 mV, respectively), suggesting that nicorandil-induced conductance is sensitive to K^+ ions and is presumably I_{KATP} . We therefore concluded that membrane potential-dependent activation of Na-Ca exchanger cannot contribute to the nicorandil-dependent $[Ca^{2+}]_i$ reduction because the compound caused little hyperpolarization in intact ventricular myocytes.

Another possible explanation of the nicorandil-induced $[Ca^{2+}]_i$ reduction is a decrease in Ca-influx through voltage-dependent Ca-channels. This seemed less likely at least with respect to the resting $[Ca^{2+}]_i$ in quiescent myocytes, where resting potentials are close to E_K and therefore negative enough to inactivate the Ca-channels. Nevertheless, we determined the effect of nicorandil on Ca-channel current, since it directly modulates $[Ca^{2+}]_i$ in the beating heart.

Inward Ca-currents are thought to be carried dominantly through a low-threshold, long-lasting (L-type) Ca-channel in guinea pig ventricular cells (30, 31). Figure 5A depicts an inward current elicited by a depolarizing voltage pulse to $+10$ mV from the holding potential of -40 mV. The inward component was attributable to the L-type Ca-channel current, because of its specific sensitivity to external Ca^{2+} ions and reversible block by the extracellular application of 2 mM $CoCl_2$ or 1 μ M nifedipine (data not shown in figure). To block the activation of I_{KATP} channels 1 μ M glibenclamide was added to the bathing solution, which caused no changes of the Ca-current. After control currents attained a steady state in a bathing Tyrode solution containing glibenclamide, subsequent addition of 0.1 mM nicorandil produced no significant changes in the inward Ca-current (Fig. 5B). Figure 5C shows peak inward current-voltage relationships in the absence and presence of nicorandil, and after wash-out. Similar failure of the nicorandil action was observed in 3 other myocytes. The mean peak inward Ca-current at $+10$ mV was 7.8 ± 1.5 pA/pF (4) in control and 7.2 ± 2.0 pA/pF (4) after nicorandil application ($P > 0.05$). These values showed a small decrease with time, but similar "rundown" was also observed in the absence of nicorandil during similar periods (~ 20 min). Thus, the nicorandil-dependent modulation in $[Ca^{2+}]_i$ does not result from its affecting L-type Ca-channel current.

DISCUSSION

Nicorandil is the prototype of pyrimidine derivative K^+ channel openers and was first shown to increase K^+ conductance in both vascular smooth and cardiac muscle (13, 22, 24, 38). Subsequent studies in both preparations also revealed the ability of the compound to stimulate soluble guanylate cyclase activity, thereby increasing intracellular cyclic GMP levels (9, 10, 19). There-

fore, nicorandil is now regarded as a unique hybrid compound with K^+ channel opening associated with the pyridyl moiety and guanylate cyclase activation generated by the nitroxy side-chain, sharing the properties of both K -channel opener and nitrovasodilator. Thus, caution is required in interpretation of the drug-induced reduction in $[Ca^{2+}]_i$ measured by fura-2 fluorescence techniques as used in the present study.

The use of the acetoxymethyl ester of fura-2 has several experimental limitations; leakage of the dye from cells, its unpredictable uptake into subcellular organelles, and its unknown hydrolysis ratio to free acid (1, 4, 17; for review, 26). Moreover, since the generation of fura-2 depends on how much of its lipophilic acetoxymethyl ester (fura-2/AM) gets into the cell, which is supposed to be hydrolyzed by endogenous cellular esterases (15), a considerable spread of fluorescence ratios (340 nm/380 nm) can be expected between the myocytes. We therefore employed percentage changes of intensity level ratio (340 nm/380 nm) as the indicator of $[Ca^{2+}]_i$. Preliminary experiments with *in vitro* Ca^{2+} calibration, however, showed that average decrease in signal ratio by 0.1 mM nicorandil corresponds to a substantial reduction of $[Ca^{2+}]_i$.

Compared to other $[Ca^{2+}]_i$ indicators such as aequorin, a photoluminescent protein, fura-2 offers reliable signals at relatively low $[Ca^{2+}]_i$ (~ 30 nM) because of its high quantum efficiency, absorption coefficient and affinity to Ca^{2+} (K_d for Ca^{2+} 140 nM; 7, 15). Such a high sensitivity for Ca^{2+} was useful in showing that nicorandil produced a consistent and reversible decrease in fluorescence intensity ratio (Figs. 1 and 2).

In an attempt to characterize the subcellular mechanism for nicorandil-dependent $[Ca^{2+}]_i$ reduction, we addressed its action as a guanylate cyclase activator (Fig. 3) because similar $[Ca^{2+}]_i$ reduction was obtained by the application of a membrane-permeable cyclic GMP analogue. Using several blockers and external Na-free condition, cyclic GMP-mediated activation of sarcolemmal Ca-pump was shown to play an important role in physiological ionic conditions. In our previous report (34), the same conclusion was deduced on ANP-dependent $[Ca^{2+}]_i$ reduction in ventricular myocytes.

These findings are in agreement with several recent biochemical studies (14, 32); cyclic GMP-dependent protein kinase (G-PK) is involved in the activation of Ca-pumping ATPase. Direct phosphorylation of the pump protein by G-PK is, however, still questionable (3).

The nicorandil action as a K -channel opener was next examined, because such an effect may also lead to a decrease in $[Ca^{2+}]_i$ by both increasing Ca-extrusion via electrogenic Na-Ca exchange and closing voltage-dependent Ca-channels. Nicorandil increased considerable K^+ conductance that was reversed by 1 μ M glibenclamide, but produced little hyperpolarization (Table I

and Fig. 4), since the resting potentials were close to E_K in quiescent ventricular myocytes. This was also confirmed during fura-2 monitoring of $[Ca^{2+}]_i$: the deprivation of extracellular Na^+ ions failed to entirely abolish the nicorandil effect.

In the final series of experiments (Fig. 5), the nicorandil action on L-type Ca-channel current was examined under conditions where Na-channel, T-type Ca-channel currents and I_{KATP} were suppressed. Peak inward current-voltage relations with or without nicorandil were superimposable, indicating that the compound did not directly affect the Ca-current. Kakei *et al.* (24) also noted that nicorandil (~ 0.2 mM) had minimal effect on the Ca-current in guinea pig ventricular myocytes. Fischmeister *et al.* (25), however, demonstrated that intracellular cyclic GMP (~ 1 μ M) could reduce the L-type Ca-channel current only when it had been previously enhanced through the cyclic AMP-dependent pathway and that its inhibition was directly mediated by the activation of G-PK.

Thus, the contribution of voltage-dependent mechanisms to reduce $[Ca^{2+}]_i$ appears to be minimal in the intact heart cells, unlike that in vascular smooth muscle cells where the resting potential is much more positive to E_K . This type of site-specificity may account for the tissue-dependent difference of the nicorandil effect (i.e., between smooth and cardiac muscles). However, it should be noted that under metabolic disturbances, where the cardiomyocytes would be greatly depolarized, nicorandil can work to restore the normal resting membrane potential by opening glibenclamide-sensitive I_{KATP} channels. More recently, I_{KATP} channels were shown to reversibly open during experimental anoxic conditions (12). In this situation, nicorandil would further facilitate I_{KATP} channel opening and act to protect against basal $[Ca^{2+}]_i$ overload through voltage-dependent actions on two major and direct $[Ca^{2+}]_i$ modulators: electrogenic Na-Ca exchanger and voltage-dependent Ca-channels. The actions of the compound in the presence of catecholamines (activation of protein kinases A and C) and metabolic failure are now under investigation.

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REFERENCES

- ALMERS, W. and NEHER, E. (1985). The Ca signal from fura-2 loaded mast cells depends strongly on the method of dye-loading. *FEBS Lett.*, **192**: 13–18.
- ARENA, J. and KASS, R.S. (1989). Enhancement of potassium-sensitive current in heart cells by pinacidil. Evidence for modulation of the ATP-sensitive potassium channel. *Cir. Res.*, **65**: 436–445.
- BALTENSPERGER, K., CARAFOLI, E., and CHIESI, M. (1988). The Ca^{2+} -pumping ATPase and the major substrates of the cGMP-dependent protein kinase in smooth muscle sarcolemma are distinct entities. *Eur. J. Biochem.*, **172**: 7–16.
- BLATTER, L.A. and WIER, W.G. (1990). Intracellular diffusion, binding, and compartmentalization of the fluorescent calcium indicators indo-1 and fura-2. *Biophys. J.*, **58**: 1491–1499.
- BOND, G.H. and HUDGINS, P. (1979). Kinetics of inhibition of Na^+ , K^+ -ATPase by Mg^{2+} , K^+ and vanadate. *Biochemistry*, **18**: 325–31.
- CHINKERS, M., GARBERS, D., CHANG, M-S., LOWE, D.G., CHIN, H., GOEDDEL, D.V., and SCHULZ, S. (1989). A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature (Lond.)*, **338**: 78–83.
- COBBOLD, P.H. and RINK, T.J. (1987). Fluorescence and bioluminescence measurement of cytoplasmic free calcium. *Biochem. J.*, **248**: 313–328.
- COOK, N.S. (1988). The pharmacology of potassium channels and their therapeutic potential. *Trends Pharmacol. Sci.*, **9**: 21–28.
- ENDO, M. and IJIMA, T. (1983). Effects of nicorandil and its interaction with isoproterenol on force of contraction and cyclic nucleotide levels of canine atrial muscle: comparison with carbachol. *J. Cardio. Pharmacol.*, **5**: 878–882.
- ENDO, M. and TAIRA, N. (1983). Relationship between relaxation and cyclic GMP formation caused by nicorandil in canine mesenteric artery. *Arch. Pharmacol.*, **322**: 319–321.
- ESCANDE, D., THURINGER, D., LEUGUERN, S., and CAVERO, I. (1988). The potassium channel opener cromakalim (BRL 34915) activates ATP-dependent K^+ channels in isolated cardiac myocytes. *Biochem. Biophys. Res. Commun.*, **154**: 620–625.
- FRIEDRICH, M., BENNDORF, K., SCHWALB, M., and HIRCHE, H.J. (1990). Effects of anoxia on K and Ca currents in isolated guinea pig cardiocytes. *Pflügers Arch.*, **416**: 207–209.
- FURUKAWA, K., ITOH, T., KAIWARA, M., KITAMURA, K., SUZUKI, H., ITO, Y., and KURIYAMA, H. (1981). Vasodilating actions of 2-nicotinamidoethyl nitrate on porcine and guinea-pig coronary arteries. *J. Pharmacol. Exp. Ther.*, **218**: 248–259.
- FURUKAWA, K-I. and NAKAMURA, H. (1987). Cyclic GMP regulation of the plasma (Ca^{2+} - Mg^{2+}) ATPase in vascular smooth muscle. *J. Biochem.*, **101**: 287–290.
- GRYNKIEWICZ, G., POENIE, M., and TSIEN, T.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**: 3440–3450.
- HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B., and SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recordings from cells and cell-free membrane patches. *Pflügers Arch.*, **391**: 85–100.
- HIGHSMITH, S., BLOEBAUM, P., and SNOWDOWNE, K.W. (1986). Sarcoplasmic reticulum interacts with the Ca^{2+} indicator precursor FURA-2/AM. *Biochem. Biophys. Res. Commun.*, **138**: 1153–1162.
- HIRAOKA, M. and FAN, Z. (1989). Activation of ATP-sensitive outward K^+ current by nicorandil (2-nicotinamidoethyl nitrate) in isolated ventricular myocytes. *J. Pharmacol. Exp. Ther.*, **250**: 278–285.
- HOLZMANN, S. (1983). Cyclic GMP as a possible mediator of coronary arterial relaxation by nicorandil (SG-75). *J. Cardiovasc. Pharmacol.*, **5**: 364–370.
- HORIE, M., IRISAWA, H., and NOMA, A. (1987). Voltage-dependent magnesium block of adenosine-triphosphate-sensitive po-

- tassium channel in guinea-pig ventricular cells. *J. Physiol. (Lond.)*, **387**: 251–272.
21. HORIE, M., SUZUKI, H., KAWAI, C., and OKADA, Y. (1989). Nicorandil reduces intracellular free Ca^{2+} level in single cardiac myocytes as revealed by fura-2 monitoring. *Circulation*, **II**: 193.
 22. IMANISHI, S., ARITA, M., KIOSUE, T., and AOMINE, M. (1983). Effects of SG-75 (nicorandil) on electrical activity of canine Purkinje fibers: Possible increase in potassium conductance. *J. Pharmacol. Exp. Ther.*, **225**: 198–205.
 23. JUNDT, H., PORTIG, H., REUTER, H., and STUCKI, J.W. (1975). The effects of substances releasing calcium ions on sodium-dependent calcium efflux from guinea-pig auricles. *J. Physiol. (Lond.)*, **246**: 229–253.
 24. KAKEI, M., YOSHINAGA, N., SAITO, K., and TANAKA, H. (1986). The potassium current activated by 2-nicotinamidoethyl nitrate (nicorandil) in single ventricular cells of guinea-pig. *Proc. R. Soc. Lond. B.*, **229**: 331–343.
 25. MÉRY, P.F., LOHMANN, S., WALTER, U., and FISCHMEISTER, R. (1991). Ca^{2+} current is regulated by cyclic GMP-dependent protein kinase in mammalian cardiac myocytes. *Proc. Natl. Acad. Sci. USA*, **88**: 1197–1201.
 26. MOORE, E.D.W., BECKER, P.L., FOGARTY, K.E., WILLIAM, D.A., and FAY, F.S. (1990). Ca^{2+} imaging in single living cells: Theoretical and practical issues. *Cell Calcium*, **11**: 157–179.
 27. MULLINS, L.J. (1979). The generation of electric currents in cardiac fibers by Na/Ca exchange. *Am. J. Physiol.*, **236**: C103–C110.
 28. MULLINS, L.J. (1981). Ion transport in heart. Raven Press, New York.
 29. NECHAY, B.R. (1984). Mechanism of action of vanadium. *Ann. Rev. Pharmacol. Toxicol.*, **24**: 501–524.
 30. NOWYCKY, M.C., FOX, A.P., and TSIEN, R.W. (1985). Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature (Lond.)*, **316**: 440–443.
 31. PELZER, D., CAVALIÉ, A., McDONALD, T.F., and TRAUTWEIN, W. (1989). Calcium channels in single heart cells. in *Isolated Adult Cardiomyocytes II* (H.M. Piper, and G. Isenberg, eds.), pp 29–73, CRC Press, Boca Raton, Florida.
 32. RASHATWAR, S., CORNWELL, T.L., LINCOLN, T.M. (1987). Effects of 8-bromo-cGMP on Ca^{2+} levels in vascular smooth muscle cells: Possible regulation of Ca^{2+} -ATPase by cGMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.*, **84**: 5685–5689.
 33. SANGUINETTI, M.C., SCOTT, A.L., ZINGARO, G.L., and SIEGEL, P.K.S. (1988). BRL 34915 (cromakalim) activates ATP-sensitive K^{+} current in cardiac muscle. *Proc. Natl. Acad. Sci. U.S.A.*, **85**: 8360–8364.
 34. TEI, M., HORIE, M., MAKITA, T., SUZUKI, H., HAZAMA, A., OKADA, Y., and KAWAI, C. (1990). Atrial natriuretic peptide reduces the basal level of cytosolic Ca^{2+} in guinea pig cardiac myocytes. *Biochem. Biophys. Res. Commun.*, **167**: 413–418.
 35. TRUBE, G., RORSMAN, P., and OHNO-SHOSAKU, T. (1986). Opposite effects of tolbutamide and diazoxide on the ATP-sensitive K^{+} channel in mouse pancreatic β -cells. *Pflügers Arch.*, **407**: 493–499.
 36. UEDA, S. and OKADA, Y. (1989). Acid secretagogues induce Ca^{2+} mobilization coupled to K^{+} conductance activation in rat parietal cells in tissue culture. *Biochim. Biophys. Acta.*, **1012**: 254–260.
 37. WILDE, A.A.M., ESCANDE, D., SCHUMACHER, C.A., THURINGER, D., MESTRE, M., and FIOLET, J.W.T. (1989). Glibenclamide inhibition of ATP-sensitive K^{+} channels and ischemia-induced K^{+} accumulation in the mammalian heart. *Pflügers Arch.* **414** (Supple 1), 176.
 38. YANAGISAWA, T. and TAIRA, N. (1980). Effect of 2-nicotinamidoethyl nitrate (SG-75) on the membrane potential of left atrial muscle fibres of the dogs. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **312**: 69–76.

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