Retardation of cation channel deactivation by mitochondrial dysfunction in adrenal medullary cells

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SECRETION OF CATECHOLAMINES in response to hypoxia is vital for the body to deal with the life-threatening event. Secreted catecholamines, especially adrenaline, increase cardiac output and enhance gluconeogenesis and glycolysis in the liver with the consequent increase in blood glucose (6). Although carotid body type I cells are a sensing site for O₂ tension, recent studies demonstrated that adrenal medullary cells are also capable of promptly detecting a decrease in O₂ tension (11, 22). Although K⁺ channel activity in isolated patch membranes was documented to be suppressed by hypoxia (11), it remains to be determined whether the hypoxia-sensitive K⁺ channel is active at resting membrane potentials and whether suppression of its activity is responsible for depolarization in response to hypoxia (2). The other is that mitochondria play a primary role for the detection (8, 9). This proposal is principally based on findings that the effects of hypoxia can be mimicked by various types of mitochondrial inhibitors.

Thompson and Nurse (25) reported that anoxia suppressed two distinct voltage-evoked K⁺ currents, Ca²⁺-dependent and delayed rectifier type, in adrenal medullary cells obtained from newborn rats. However, a depolarizing response or receptor potential to anoxia was not suppressed by bath addition of 10 mM tetraethylammonium, which completely suppressed the anoxia-sensitive K⁺ currents. On the other hand, Mojet et al. (24) reported that the depolarization of mitochondrial membrane potential preceded an increase in [Ca²⁺]i in response to hypoxia in the rat chromaffin cell, and they proposed that mitochondria can serve as a site for detection of a decrease in O₂. Consistent with this hypothesis, cyanide (CN) and anoxia induced activation of a nonselective cation (NS) channel and inhibition of the Na⁺ pump in guinea pig adrenal chromaffin cells (15). This cation channel may be the same as that activated by muscarinic receptor stimulation (19), because muscarine failed to induce a further inward current during the full production of a current in response to CN (14). If this notion is tenable, then anoxia and CN might activate the channel through phosphorylation because the muscarinic activation of channels and deactivation may be mediated by a protein kinase and a Mg²⁺-dependent phosphatase, respectively (16, 20). In the present experiment, we examined the mechanism for CN activation of NS channels. Our findings are consistent with the notion that exposure to CN diminishes the deactivation process with the consequent dominance of the activation.

METHODS

Whole cell recordings. Experiments on dissociated adrenal medullary cells were done, as described elsewhere (17). Briefly, female guinea pigs weighing 250–300 g were killed by a blow to the neck, and the adrenal glands were eliminated and immediately put into ice-cold Ca²⁺-free solution in which 1.8 mM Ca²⁺ was simply removed from a standard saline containing (mM): 137 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 0.53 NaH₂PO₄, 5 D-glucose, 5 HEPES, and 4 NaOH. Adrenal medullas were cut into three to six pieces and incubated for 30 min with 0.25% collagenase dissolved in the Ca²⁺-free
solution. After the incubation, the tissues were washed three times in the Ca²⁺-free solution and then kept in it at room temperature (23–25°C). A few pieces of the tissues were put in the bath apparatus, which was placed on an inverted microscope, and adrenal chromaffin cells were dissociated mechanically with fine needles. Then, dissociated cells were allowed to adhere to the bottom for a few minutes before the bath apparatus was perfused with saline at a rate of 1 ml/min. The whole cell current was recorded with the perforated-patch method (13). The current was recorded with an Axopatch 200A amplifier (Axon Instruments) and then fed into a brush recorder after low-pass filtering at 3 or 5 Hz and into a video tape after being digitized with an analog-to-digital converter. The pipette solution contained (mM): 120 potassium isethionate, 20 KCl, 10 NaCl, 10 HEPES, and 2.6 KOH. On the day of the experiment, nystatin dissolved in dimethyl sulfoxide (10 mg in 50 µl) was added to the pipette solution at a final concentration of 100 µg/ml. Glucose and NaCl in the standard saline were equimolarly replaced with sucrose and NaCN in a CN solution. The pH of the pipette solution and external solutions was adjusted to 7.2 and 7.4 with KOH and NaOH, respectively. All chemicals were bath applied, and a CN-induced current (I_CN) was evoked by perfusion with the CN solution or 3 µM muscarine-containing solution, unless otherwise noted. The membrane potential was corrected for a liquid junction potential of ~12 mV between the pipette solution and the standard solution. Experiments except for those at low temperatures were carried out at 23–25°C. When bath temperature was lowered from room temperature, the perfusate was cooled with a Peltier device. Thus the temperature around the cell examined was expected to be lower than that at the outlet, where temperature was measured. In a separate experiment, the temperature at the center of bath was lower by ~3°C than the 19.7°C at the outlet, by 1.6°C than the 20.8°C, and by 1.4°C than the 21.8°C. Data are expressed as means ± SD, and statistical significance was determined with Student’s t-test.

Fluorescence recordings. To label the cell surface with a fluorescence dye, we incubated dissociated cells for 30 min in a standard solution to which 5 µM di-8-ANEPPS and 0.05% Pluron F-127 were added. The dish in which the cells settled was placed on a Zeiss Axiovert microscope attached to a Zeiss LSM 410 laser confocal scanning unit (Carl Zeiss). The objective lens was an oil-immersion lens with a magnification of ×63 and a numerical aperture of 1.25. Illumination with 488 nm was provided by an argon laser, and emission was monitored above 570 nm because the peak emission wavelength was reported to be 570–580 nm (4). The theoretical spatial resolution given by the equation $d = \lambda/(2 \times NA)$, where $d$ represents the smallest resolvable distance, $\lambda$ is the wavelength of emission light (~580 nm), and NA is the numerical aperture (1.25), is ~0.2 µm. Thus images and line-scan images were obtained in all experiments with a pixel size of <0.2 µm and with a full width at half-maximal intensity of ~0.7 µm. The actual value of $d$ may be twice or three times larger than the theoretical one because of diffraction (23). To study effects of CN, one-half of the 2-ml solution in the dish was replaced with the CN solution and the administration was completed within 8 s.

Chemicals. Nystatin, (E)-muscarine chloride, and Pluron F-127 were obtained from Sigma; di-8-ANEPPS was from Molecular Probes; N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA-1004) was from Seikagaku (Tokyo, Japan); collagenase was from Yakult (Tokyo, Japan); NaCN was from Hayashi Pure Chemical (Tokyo, Japan).

RESULTS

Retention of $I_M$ deactivation by CN. One of the findings for involvement of phosphorylation in the activation of NS channels is a reversible suppression of the muscarinic production of a nonselective cation current ($I_{NS}$) by protein kinase inhibitors (16, 18). Consistent with the previous result, addition of 300 µM HA-1004, a general protein kinase inhibitor (12), to a 3-µM muscarine-containing perfusate resulted in a gradual decline of $I_M$ in a reversible manner, whereas the inhibitor failed to suppress $I_{CN}$ (Fig. 1A). In the previous experiment (15), 57 and 43% of $I_{CN}$ were estimated to be due to activation of NS channels and...
inhibition of the Na⁺ pump current (I_pump), respectively. I_CN in this particular cell, however, might have comprised only inhibition of the I_pump, but this possibility may not be feasible because the subsequent application of 3 and 10 µM muscarine did not induce a further inward current, as was noted previously. In a total of eight cells, HA-1004 suppressed I_M in a concentration-dependent manner but did not affect I_CN (Fig. 1B). This finding raises the possibility that exposure to CN activates NS channels independent of phosphorylation. One of such possibilities is that a change in membrane tension is responsible for the NS channel activation, because hypoxia and metabolic suppression were reported to increase volume in various types of cells (10, 21). Thus we investigated whether or not a short exposure to CN would induce any alteration in cell size. To this end, the cell surface was labeled with di-8-ANEPPS and fluorescence was observed in line-scan or z-axis images. Figure 2 shows results of line-scan analysis. The line marked in the image (Fig. 2A) was scanned every 0.1 s, and these scans were used to construct the line-scan image (Fig. 2B). Addition of 1 ml CN solution to 1 ml of dish solution (final CN concentration, 2.5 mM) did not induce any change in cell diameter for 200 s in nine of ten cells tested, as noted in one cell (Fig. 2, A and B, left). However, in another cell (Fig. 2, A and B, right), the diameter began to increase 26 s after the onset of CN exposure and the increase continued. Figure 2C summarizes relative diameters of 10 cells during 2-min exposure to CN. It is evident that short exposure to CN did not alter cell size. Similarly, z-axis images were not altered during the CN exposure (not shown). The results indicate that short application of CN does not induce an increase in diameter at least in the order of ~0.5 µm.

If phosphorylation is indeed involved, then the activation of channels might be attributed to a decrease in phosphatase activity with the consequent dominance of kinase activity. If this is the case, then I_M should diminish slowly after washout of muscarine while I_CN is also active. This inference was examined by applying muscarine at a maximum of the current evoked by 0.5 mM CN; thus I_M was expected to be reversible under such conditions because the amplitude of the fully developed I_CN was about one-half of I_M and activation of the channel may not have been saturated (14). Figure 3A shows that deactivation of I_M after washout was considerably retarded in I_CN production, an effect that rapidly disappeared after washout of CN. To elucidate the relation between retardation of I_M deactivation and I_CN production, half-decay times of I_M in the production of I_CN were expressed as a fraction of those of control I_M in the same cells and the ratio was plotted against the relative amplitude of I_CN, which had developed on muscarinic stimulation. The relative amplitude of I_CN was expressed as a fraction of I_M evoked in the absence of CN. Figure 3B shows that as the relative amplitude of I_CN increased, deactivation of I_M slowed. Furthermore, the amplitude of I_M evoked in the presence of CN diminished with an increase in the relative amplitude of I_CN (not shown).

Effects of low temperature. The foregoing observations suggest that suppression of mitochondrial function retards the deactivation of I_M. We then asked if low temperatures have a similar retarding action on I_M, because mitochondrial F$_1$-ATPase activity diminished markedly below ~20°C (1, 7). First, to determine how low temperature affects mitochondrial function, restoration of the Na⁺ pump activity from CN inhibition was examined. The I_CN elicited in the presence of 6 mM Ba$^{2+}$
was in a major part attributed to inhibition of $I_{\text{pump}}$, and suppression of the pump activity by CN was thought to be due to a decrease in intracellular ATP contents (15). Thus, if ATP production in mitochondria is indeed impaired by a decrease in temperature, then restoration of the pump activity should be slow. Figure 4 shows that this is the case. As the temperature in the outflowing perfusate increased from 19.6°C (actual temperature around the cell, ~16.6°C), restoration of the pump activity became rapid (Fig. 4A). This rapid event could not be ascribed to a general effect of temperature on kinetics. The time required for half-suppression of $I_{\text{pump}}$ after CN administration increased only slightly with decreasing temperature, whereas the time for half-restoration after washout increased steeply (Fig. 4B).

Furthermore, the temperature dependence of half-times for production and diminution of $I_{\text{CN}}$ in the absence of Ba$^{2+}$ did not apparently differ from those for $I_{\text{CN}}$ in Ba$^{2+}$ (Fig. 4B). In Fig. 4C, amplitudes of $I_{\text{CN}}$ in the presence and the absence of 6 mM Ba$^{2+}$ were plotted against temperature. The apparent $x$-intercepts for $I_{\text{CN}}$ with and without Ba$^{2+}$ were 18.7 and 17.8°C, respectively. Because restoration of the pump activity is likely to reflect that of mitochondrial functions, a decrease in temperature below 21°C (actual temperature, ~19.5°C) may impair the restoration.

Whether or not deactivation of $I_{\text{M}}$ is retarded at low temperatures was then examined. $I_{\text{M}}$ was successively evoked as the temperature in the outflow decreased from 28.5 to 15.7°C and then increased up to 29°C (Fig. 3).
It is evident that the half-decay time markedly increased with decreasing temperature below 21°C (Fig. 5C), whereas the half-rise time only slightly increased. A similar difference in temperature dependence of the half-rise time and the half-decay time (Fig 5, B and C) of I_CN was noted below 21°C. The half-rise times of the first two I_CNs elicited at 28.5 and 22.7°C were apparently smaller; these results, however, may not indicate the marked temperature dependence of the half-rise time, because results of I_CN elicited at 22.2 and 26.4°C in the increasing phase of temperature did not differ from those of I_CN below 21°C. The time course of the developing process of I_CN sometimes slowed with time of the recording and then became stable. A comparison of temperature dependence of the decay time for I_M with that for I_CN reveals that the decay process of I_M and I_CN had the same temperature dependence. In Fig. 5D, amplitudes of I_M and I_CN are plotted against temperature. The regression line for I_CN had a steeper slope than that for I_M, and the apparent x-intercepts for I_M and I_CN were 9 and 13°C, respectively.

To facilitate analysis of temperature effects in different cells, slopes and x-intercepts for I_CN in the presence of 6 mM Ba^{2+} and for I_M were expressed as fractions of those for I_CN in its absence in the same cells. The relative x-intercept of I_CN in the presence of Ba^{2+} and that of I_M thus obtained significantly differed from one, and similarly, the relative slope of I_M was noticeably different from one (Fig. 6, A and B). These results indicate that I_pump inhibition and I_NS, which constitute I_CN, have a different temperature dependence. On the other hand, ratios of half-decay times at 20°C (actual temperature, ~17°C) to those at 24°C for I_CN in the presence of Ba^{2+} and for I_M did not differ appreciably, compared with those for I_CN in its absence, thereby suggesting the involvement of a common mechanism for the retardation of both restoration of pump activity and diminution of I_NS.

**DISCUSSION**

Our previous study (15) suggested that 57 and 43% of 5 mM CN-induced inward currents can be attributed to activation of NS channels and inhibition of the I_pump, respectively. Because amplitude of I_NS diminished progressively in the absence of Na^{+} pump activity and this decrease was enhanced by replacement of sucrose with glucose, i.e., by glycolytic production of ATP, activation of the channel was presumed to be due to an ATP decrease that resulted from consumption by energy-
dependent processes, such as Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. Thus, to obtain a maximum stimulation of NS channels, CN activation of the channel was observed as I\textsubscript{CN} without isolation of the I\textsubscript{NS}. In the present experiment, the notion that I\textsubscript{CN} comprises production of I\textsubscript{NS} and inhibition of the Na\textsuperscript{+} pump was further supported by a difference in temperature dependence of I\textsubscript{M} and I\textsubscript{pump} inhibition. The relative values of x-intercept for I\textsubscript{M} and I\textsubscript{pump} inhibition significantly differ from one. Similarly, I\textsubscript{M} production and I\textsubscript{pump} inhibition had relative slopes of 0.82 and 1.05, respectively, and the former appreciably differed from one. Thus temperature dependence of I\textsubscript{CN} is thought to reflect a combination of those related to I\textsubscript{M} production and I\textsubscript{pump} inhibition.

In carotid body type I cells, a decrease in O\textsubscript{2} tension was proposed to be sensed by an O\textsubscript{2}-sensitive K\textsuperscript{+} channel or its closely associated regulator (11, 22) or by mitochondria (8, 9). Similarly, these two mechanisms were suggested to be involved in O\textsubscript{2} sensing in adrenal medullary cells (14, 24, 25). The findings that CN-induced activation of NS channels and inhibition of the Na\textsuperscript{+} pump were reproduced by exposure to anoxia are consistent with the mitochondria hypothesis. The rapid secretion of catecholamines by hypoxia would be accounted for readily by the membrane ion channel hypothesis, whereas how the dysfunction of mitochondria promptly induces a change in membrane excitability would be a challenging issue for the mitochondrial hypothesis. The sequence of our experiments revealed that dysfunction of mitochondria indeed induces a rapid depolarization through inhibition of the Na\textsuperscript{+} pump and activation of NS channels. In particular, anoxia or CN activation of NS channels is estimated to correspond to ~60% of anoxia- or CN-induced currents and exposure to anoxia or CN even in the absence of Na\textsuperscript{+} pump activity results in activation of the channel. Thus mechanisms for stimulation of NS channels by anoxia or chemical hypoxia seem to develop specially for transduction of O\textsubscript{2} signal to catecholamine secretion. The present results suggest that CN activation of NS channels is due to suppression of the deactivation process for the channel. First, the diminution of I\textsubscript{M} after washout was retarded in I\textsubscript{CN} generation, and the degree of retardation depended on the relative production of I\textsubscript{CN} on muscarinic stimulation. As the relative amplitude of I\textsubscript{CN} compared with that of I\textsubscript{M} increased, I\textsubscript{M} diminution was even more retarded. This close correlation between relative production of I\textsubscript{CN} and retardation of I\textsubscript{M} decay indicates that the deactivation process for the NS channel diminishes in generation of I\textsubscript{CN}. Because the biophysical properties of CN or anoxia-sensitive channels resemble those of the muscarinic one and the muscarinic stimulation of the channel was occluded in an amplitude-dependent manner by generation of I\textsubscript{CN} (Figs. 1 and 3; Ref. 13), there would be no doubt that exposure to CN or hypoxia activates the same NS channel as that regulated by the muscarinic receptor. Thus it is likely that diminished deactivation process for NS channels is responsible for CN activation of the channel. The decrease in rate constant for deactivation is expected to shift the equilibrium between activation and deactivation toward the former with the consequent production of I\textsubscript{NS}. Secondly, the failure of HA-1004 to suppress I\textsubscript{CN} is consistent with our hypothesis. The muscarinic activation of NS channels was reversibly suppressed by various isoquinoline sulfonamide derivatives with different potencies (16, 18), and this inhibition was assumed to be due to inhibition of the protein kinase involved and the consequent shift of equilibrium between phosphorylation and dephosphorylation toward the latter (20). The fact that HA-1004 failed to suppress I\textsubscript{CN} suggests that the rate constant for deactivation decreased almost to null during exposure to 5 mM CN. Thirdly, deactivation of I\textsubscript{M} was retarded at low temperatures. This low-temperature effect is probably due to the dysfunction of mitochondria, because restoration of the Na\textsuperscript{+} pump activity from CN inhibition was retarded below 21°C.
The fact that metabolic inhibition with CN and low temperatures induce a similar retardation of \( I_{N} \) deactivation indicates that the deactivation process of NS channels is closely associated with the mitochondrial function. Based on three lines of evidence, it would be rational to assume that exposure to CN or anoxia activates NS channels through suppression of the mitochondrial function. Our previous studies (16, 20) suggest that a Mg\(^{2+}\) -dependent phosphatase is responsible for deactivation of the NS channel. This thesis was not examined directly in the present experiment, because the Mg\(^{2+}\) -dependent phosphatase has not been identified molecularly and there is no specific inhibitor for protein phosphatase IIC (5), a candidate for the phosphatase involved. The best means to inhibit the phosphatase activity is removal of Mg\(^{2+}\).

In preliminary experiments, we found that the CN potency to induce an inward current was almost abolished by decreasing concentrations of free Mg\(^{2+}\) inside the cell.

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