Current Topics in Pharmacology
Vol. 20, 2016

Original Communication

Mitochondrial membrane potential oscillations in isolated cardiomyocytes as revealed by fluorescence microscopy

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ABSTRACT

Mitochondrial membrane potential oscillation was studied in isolated rat and guinea pig ventricular cardiomyocytes using fluorescence microscopy. Spontaneous oscillation of tetramethylrhodamine ethylester (TMRE) fluorescence occurred in the time scale of seconds; the oscillation was abolished by chelation of intracellular Ca^{2+}. The TMRE fluorescence of each mitochondria unit changed independently, and a drop in TMRE fluorescence was preceded by a prolonged rise in fluo-4 fluorescence. Spontaneous oscillation of flavoprotein fluorescence in the second-to-minute time scale was observed in individual mitochondria units, which was abolished under chelation of intracellular Ca^{2+} and was followed by a decrease in TMRE fluorescence. The oscillation of TMRE fluorescence was markedly inhibited by inhibitors of mitochondrial electron transport or the permeability transition pore. These results indicate that fluctuations in intracellular Ca^{2+} and electron transport activity may cause fluctuations in mitochondrial membrane potential.

KEYWORDS: mitochondrial membrane potential, cardiomyocytes, fluorescence microscopy, intracellular Ca^{2+}, flavoprotein

INTRODUCTION

Myocardial contraction and the underlying regulation of intracellular Ca^{2+} are largely dependent on adenosine triphosphate (ATP) produced by aerobic energy metabolism of the mitochondria [1]. The potential gradient across the mitochondrial inner membrane generated by the electron transport system is the driving force for ATP biosynthesis [2]. Increase in the amplitude of the Ca^{2+} transient, which is the major determinant of myocardial contractile force [3], is also considered to activate mitochondrial energy metabolism; increased mitochondrial Ca^{2+} causes an increase in the enzymes involved in the tricarboxilic acid (TCA) cycle and electron transfer [4-6]. Thus, intracellular Ca^{2+} appears to serve as a signal to enhance myocardial contraction under positive inotropic stimuli and at the same time, elevate energy metabolism to meet the increased energy demands of the cardiomyocytes.

Under myocardial ischemia, reduced electron transport causes loss of the mitochondrial membrane potential and leads to a decline in ATP synthesis and contractile force. This is accompanied by elevation of the cellular Ca^{2+} content above controllable level, a situation known as Ca^{2+} overload [7]. The elevation of the basal cytoplasmic Ca^{2+} concentration and local oscillations of Ca^{2+} such as Ca^{2+} waves which occur in the Ca^{2+} overloaded cardiomyocytes, are not only arrhythmogenic but also lead to long-term deterioration of cellular function. One of the major implications of Ca^{2+} overload is mitochondrial dysfunction; excess Ca^{2+} causes irreversible mitochondrial damage and leads to a loss of ATP synthesis [8-10]. Thus, mitochondrial Ca^{2+} is closely and intricately related to mitochondrial function.

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Oscillation of the mitochondrial membrane potential has been repeatedly observed in various cell types and in isolated mitochondria [11-15]. Although this oscillation appears to be an interesting model which would provide information on the regulation of mitochondrial membrane potential including the role of Ca^{2+}, its precise mechanism and biological significance remain to be clarified. In the present study, we applied rapid scanning confocal and epifluorescence microscopy on isolated cardiomyocytes. We observed that individual mitochondria units had their own time course of membrane potential oscillation in the time scale of seconds. We also examined the effects of pharmacological interventions.

MATERIALS AND METHODS

General

All experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Preparation of cardiomyocytes

Ventricular myocytes were prepared from rat and guinea pig hearts by Langendorff perfusion and collagenase digestion as in our previous study [9], and were plated on coverslips attached to an experimental chamber. They were superfused with Tyrode's solution of the following composition (mM): NaCl 143, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 1.8, NaH_2PO_4 0.33, glucose 5.5 and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 5.0 (pH 7.4). The solution was gassed with 100% O_2 and warmed to 37 °C. Only the cells with conspicuous cross-striation and responsiveness to electrical field stimulation were used in the experiments. Chelation of intracellular Ca^{2+} was performed by loading the cells with 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA); cells were superfused with Ca^{2+}-free extracellular solution containing O,O'-Bis(2-aminoethyl)ethyleneglycol-N,N,N',N'-tetraacetic acid (GEDTA) (3 mM) and the acetoxymethyl ester of BAPTA (BAPTA-AM; 100 mM).

Probe loading

For the measurement of mitochondrial membrane potential, the cells were loaded with tetramethyl-rhodamine ethylester (TMRE) by placing them in an extracellular solution containing TMRE (100 nM) for 15 to 20 min at room temperature. For the measurement of cytoplasmic Ca^{2+}, the cells were loaded with fluo-4 by exposing them to extracellular solution containing fluo-4-AM (5 μM) for 30 min at 37 °C. After completion of probe loading, the chamber was placed on a fluorescence microscope, and the cells were superfused with the Tyrode's solution at 37 °C.

Confocal microscopy

Confocal microscopy was performed with LSM 5-*LIVE* or LSM 510 *META* (Carl Zeiss, Oberkochen, Germany) as in our previous study [16]. The objective lens was x40 Plan Apochromat water immersion type. For TMRE fluorescence, the excitation wavelength was 543 nm and fluorescence with wavelength above 560 nm was detected and analyzed. For imaging of fluo-4 and flavoprotein fluorescence, the excitation wavelength was 488 nm and fluorescence within the wavelength range of 500 nm to 530 nm was detected and analyzed. The scanning speed was from1-frame/36 ms to 1-frame/160 ms.

Epifluorencence microscopy

Epifluorescence microscopy was performed with an inverted microscope Olympus IX 70 (Olympus, Tokyo, Japan) and Aquacosmos analyzing system (Hamamatsu Photonics, Hamamatsu, Japan) as in our previous study [9]. The objective lens was x40 Plan Apochromat water immersion type. TMRE-loaded cells were excited at 543 ± 10 nm with a Xenon Lamp, and fluorescence with wavelength above 610 nm was detected and analyzed.

Drugs and chemicals

TMRE was purchased from Life Technologies (Waltham, MA, USA), fluo-4-AM, GEDTA and BAPTA-AM from Dojindo Laboratories (Kumamoto, Japan), and cyclosporine A from Sigma-Aldrich (St. Louis, MO, USA), NaCN from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All the chemicals used were of the highest commercially available quality.

RESULTS

Oscillation of the mitochondrial membrane potential in cardiomyocytes

In guinea pig ventricular cardiomyocytes loaded with the mitochondrial membrane potential probe

TMRE, spontaneous oscillation in mitochondrial fluorescence intensity was observed (Fig. 1). A drop and recovery of total cellular intensity was repeatedly observed in the time scale of seconds. Such an oscillation of TMRE fluorescence was totally absent in cells loaded with a Ca^{2+} chelator, BAPTA.

Measurement of membrane potential in mitochondria units

Analysis of mitochondria units by confocal microscopy revealed that the TMRE fluorescence intensity of each mitochondria unit changed independently and that the change was more of an all-or-none fashion compared with that of the whole cellular intensity (Fig. 2). Simultaneous measurement of cytoplasmic Ca^{2+} and mitochondrial membrane potential was performed in cells loaded with fluo-4 and TMRE. Rise in cytoplasmic Ca^{2+} in the form of a propagating Ca^{2+} wave was followed by a drop in TMRE fluorescence. Such a phenomenon occurred after repeated and/or prolonged elevation of cytoplasmic Ca^{2+}.

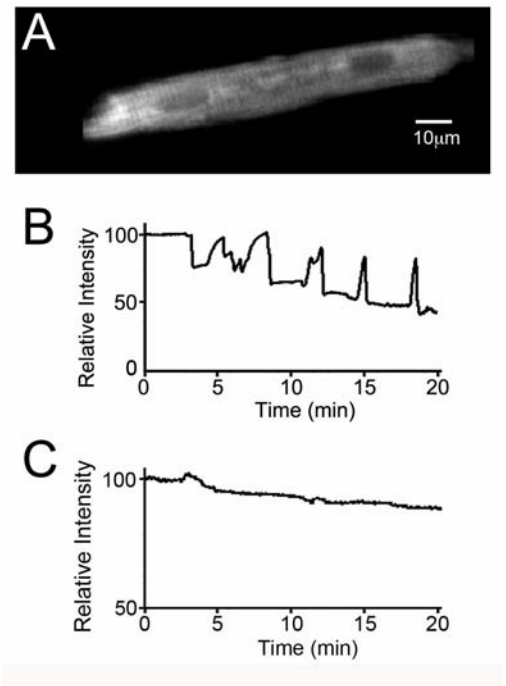

Fig. 1. Spontaneous oscillation of mitochondrial membrane potential. **A:** An epifluorescence image of TMRE fluorescence in a guinea pig cardiomyocyte. **B:** Time course of the cellular total TMRE fluorescence in the cell shown in A. **C:** Time course of TMRE fluorescence in a guinea pig cardiomyocyte treated with BAPTA.

Measurement of mitochondrial electron transport activity and membrane potential

Measurement of mitochondrial electron transport activity was performed with cellular flavoprotein fluorescence. Spontaneous oscillation in fluorescence intensity was observed in the time scale of seconds; analysis of multiple cellular regions revealed that the fluorescence intensity of each region changed either independently or in synchronization (Fig. 3A). Oscillation of flavoprotein fluorescence was not observed in cells superfused with Ca^{2+}-free extracellular solution (Fig. 3B). Simultaneous measurement of flavoprotein fluorescence and mitochondrial membrane potential in cells loaded with TMRE revealed that, in individual mitochondria units, the decrease in TMRE fluorescence was preceded by an increase in flavoprotein fluorescence (Fig. 3C).

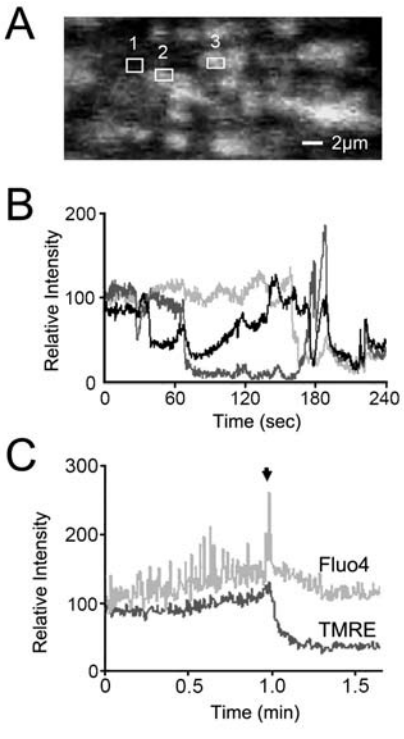

Fig. 2. Simultaneous measurement of mitochondrial membrane potential and cytoplasmic Ca^{2+}. **A:** A confocal image of TMRE fluorescence obtained in a rat cardiomyocyte. White squares 1 to 3 indicate the regions for quantification. **B:** Time course of the changes in TMRE fluorescence in the regions indicated in A. **C:** Time course of the changes in fluo-4 fluorescence (gray) and TMRE fluorescence (black) in a single mitochondria unit. The arrow indicates passage of a Ca^{2+} wave.

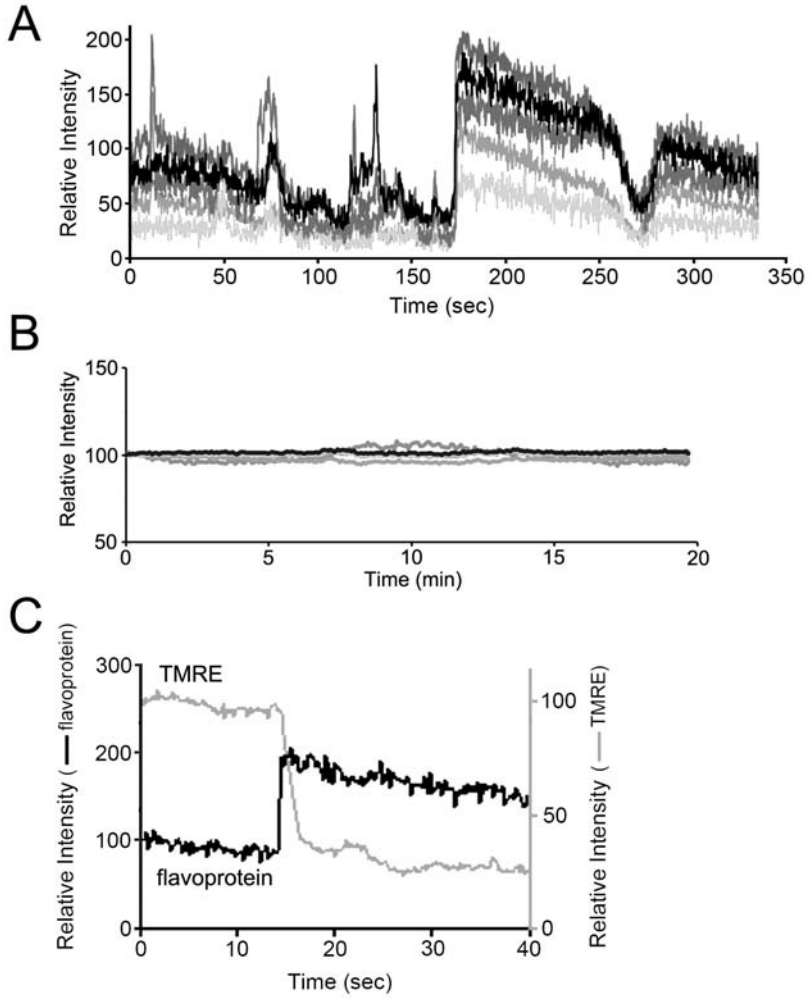

Fig. 3. Measurement of flavoprotein fluorescence and mitochondrial membrane potential. **A:** Time course of the changes in flavoprotein fluorescence of five different regions in a guinea pig cardiomyocyte. **B:** Time course of flavoprotein fluorescence in five different regions in a guinea pig cardiomyocyte treated with BAPTA. **C:** Time course of the changes in flavoprotein fluorescence (black) and TMRE fluorescence (gray) in a guinea pig cardiomyocyte loaded with TMRE.

Effects of pharmacological interventions on mitochondrial membrane potential

Effects of pharmacological agents related to mitochondrial electron transport on mitochondrial membrane potential were examined in cells loaded with TMRE. The spontaneous oscillation in TMRE fluorescence (Fig. 4A) was markedly inhibited by treatment with NaCN (Fig. 4B) and cyclosporin A (Fig. 4C).

DISCUSSION

The present study was undertaken to obtain information on the mechanisms for the oscillation of mitochondrial membrane potential in isolated cardiomyocytes. Spontaneous oscillation in mitochondrial membrane potential was detected by TMRE fluorescence at the whole cell level (Fig. 1) and mitochondria unit level (Fig. 2). The oscillation in each mitochondria unit was independent and the depolarization was reversible. This indicates that fluctuation in some factor within the local environment is causing reversible changes in the mitochondria. Simultaneous measurement of cytoplasmic Ca^{2+} and the mitochondrial membrane potential revealed that depolarization of the mitochondrial membrane is preceded by an increase

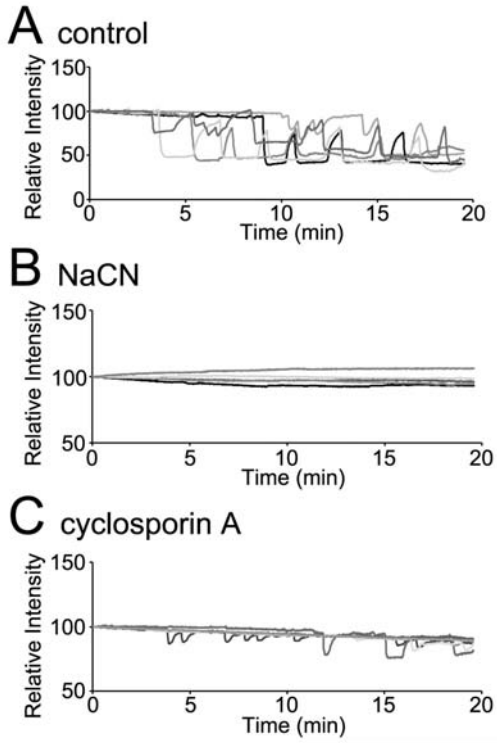

Fig. 4. Effect of pharmacological agents on mitochondrial membrane potential oscillation. **A:** Time course of TMRE fluorescence obtained in guinea pig cardiomyocytes untreated with pharmacological agents. **B:** Time course obtained in the presence of NaCN. **C:** Time course obtained in the presence of cyclosporin A. The 5 lines in each panel indicate total cellular TMRE fluorescence obtained from 5 different cells.

in cytoplasmic and intramitochondrial Ca^{2+}. Absence of oscillation in BAPTA-treated cells indicates the causal relationship between Ca^{2+} and mitochondrial membrane potential oscillation. This agrees with an earlier report with rat cardiomyocytes showing that focal increase in cytoplasmic Ca^{2+} caused by release from the sarcoplasmic reticulum (SR) causes focal depolarization of the mitochondrial membrane potential [17].

The relationship between cytoplasmic Ca^{2+} and depolarization appeared not so straightforward. Exposure of the mitochondria to prolonged and/or marked elevations in cytoplasmic Ca^{2+} caused depolarization of the mitochondrial membrane (Fig. 2C). Conflicting findings have been reported concerning whether the action potential-evoked Ca^{2+} transients can cause a beat-to-beat variation in mitochondrial Ca^{2+} [18-20]. Although entry of Ca^{2+}, as a divalent cation, into the mitochondria itself is a depolarizing factor, the non-linear relationship between mitochondrial Ca^{2+} and depolarization suggests the presence of some additional regulatory mechanism.

To obtain information on the mechanisms connecting intramitochondrial Ca^{2+} to depolarization, we measured flavoprotein fluorescence as an index of mitochondrial electron transport activity (Fig. 3). The observed local fluctuations in flavoprotein fluorescence indicated that electron transport activity is under the influence of local cytoplasmic conditions and differs among mitochondria units. The similarity of the time course of the fluctuations of flavoprotein fluorescence and TMRE fluorescence suggested a functional relation. Simultaneous measurements revealed that an increase in flavoprotein fluorescence is indeed accompanied by a subsequent decrease in mitochondrial membrane potential. This is consistent with an earlier observation that the cellular dispersion of TMRE fluorescence correlates with flavoprotein fluorescence [21]. Inhibition of fluctuation in TMRE fluorescence by the electron transport inhibitor NaCN (Fig. 4B) provides pharmacological evidence for causal relationship between electron transfer activity and membrane potential fluctuation.

The mitochondrial electron transport is directly coupled to extrusion of proton from the matrix across the mitochondrial inner membrane which in principle causes hyperpolarization of the mitochondrial membrane [22]. Thus, the observation that increases in intramitochondrial Ca^{2+} and flavoprotein fluorescence intensity precede mitochondrial depolarization suggests the presence of mechanisms which links electron transport activity to membrane depolarization. Elevated electron transport activity is known to cause various cellular reactions including opening of the permeability transition pore on the mitochondrial inner membrane [23]. This provides a probable explanation for the temporal correlation between electron transport activity and mitochondrial depolarization. Indeed, treatment with the permeability transition pore inhibitor, cyclosporine A, markedly inhibited fluctuation in TMRE fluorescence (Fig. 4C).

Increase in mitochondrial Ca^{2+} appears to have multiple effects on the mitochondrial membrane potential depending on the cellular condition [10].

Under positive inotropic stimuli, increased mitochondrial Ca^{2+} can cause membrane hyperpolarization through activation of the electron transfer system, which serves as a mechanism to enhance ATP synthesis to meet the increased energy demand of the working cardiomyocyte. However, under pathological conditions such as myocardial ischemia, mitochondrial Ca^{2+} overload rather causes mitochondrial depolarization and a long-term deterioration of mitochondrial function including loss of ATP synthesizing capacity. The present results imply that mitochondrial Ca^{2+} can trigger a rapid and transient mitochondrial depolarization through opening of the permeability transition pore. This can possibly serve as a negative feedback mechanism to inhibit further progression of mitochondrial Ca^{2+} overload. This self-protective mechanism operating at the individual mitochondria unit level may lead to long-term attenuation of the overall cellular damage under severe conditions such as myocardial ischemia.

CONCLUSION

Spontaneous oscillation of membrane potential in the time scale of seconds occurs in mitochondria units of isolated cardiomyocytes from the rat and guinea pig. Fluctuations in intracellular Ca^{2+} and electron transport activity may cause fluctuations in mitochondrial membrane potential through transient opening of the permeability transition pore.

ACKNOWLEDGMENTS

This study was supported in part by Grants-in-Aid for Scientific Research from the ministry of Education, Culture, Science, and Technology of Japan to I.N. (25860194) and H.T. (15K08247).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest concerning this study.

REFERENCES

1. Stanley, W. C. 2002, Heart Failure Rev., 7, 115.
2. Skarka, L. 2002, Physiol. Res., 51, 425.
3. Tanaka, H., Namekata, I., Nouchi, H., Shigenobu, K., Kawanishi, T. and Takahara, A. 2009, J. Pharmacol. Sci., 109, 327.
4. Ichas, F. 1997, Cell, 89, 1145.
5. Balaban, R. S. 2009, Biochim. Biophys. Acta, 1787, 1334.
6. Griffiths, E. J. and Rutter, G. A. 2009, Biochim. Biophys. Acta, 1787, 1324.
7. Talukder, M. A. H., Zweier, J. L. and Periasamy, M. 2009, Cardiovas. Res., 84, 345.
8. Namekata, I., Nakamura, H., Shimada, H., Tanaka, H. and Shigenobu, K. 2005, Life Sci., 77, 312.
9. Namekata, I., Shimada, H., Kawanishi, T., Tanaka, H. and Shigenobu, K. 2006, Eur. J. Pharmacol., 543, 108.
10. Gustafsson, A. B. and Gottlieb, R. A. 2008, Cardiovas. Res., 77, 334-343.
11. Cortassa, S. 2004, Biophys. J., 87, 2060.
12. Smaili, S. S. 1999, Cell Calcium, 26, 121.
13. Vergun, O. 2003, Biophys. J., 85, 3358.
14. O'Reilly, C. M., Fogarty, K. E., Drummond, R. M., Tuft, R. A. and Walsh, J. V. Jr. 2004, Am. J. Physiol. Cell. Physiol., 286, C1139.
15. Hattori, T. 2005, Cell, 82, 415.
16. Iida-Tanaka, N., Namekata, I., Kaneko, M., Tamura, M., Kawanishi, T., Nakamura, R., Shigenobu, K. Tanaka, H., 2007, J. Pharmacol. Sci., 104, 397.
17. Duchen, M. R. 1998, J. Cell Biol., 142, 975.
18. Isenberg, G., Han, S., Schiefer, A. and Wendt-Gallitelli, M. F. 1993, Cardiovasc. Res., 27, 1800.
19. Chacon, E., Ohata, H., Harper, I. S., Trollinger, D. R., Herman, B. and Lemasters, J. J. 1966, FEBS Lett., 382, 31.
20. Di-Lisa, F., Gambassi, G., Spurgeon, H. and Hansford, R. G. 1993, Cardiovasc. Res., 27, 1840.
21. Romashko, D. N., Marban, E. and O'Rourke, B. 1998, Proc. Natl. Acad. Sci. USA, 95, 1618.
22. Poburko, D. and Demaurex, N. 2012, Pflugers Arch. Eur. J. Physiol., 464, 19.
23. Bernaldi, P. 1999, Physiol. Rev., 79, 1127.