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DESENSITIZATION TO NOREPINEPHRINE INCLUDES REFRACTORINESS OF CALCIUM RELEASE IN MYOCARDIAL CELLS

Paul Erne and Kent Hermsmeyer*

Cardiovascular Research Laboratory, Chiles Research Institute, Providence Medical Center, and Oregon Health Sciences University, Portland, OR

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Localization of myoplasmic free calcium was measured in fura2-loaded single rat myocardial cells to determine whether the mechanism of norepinephrine desensitization includes redistribution of calcium. Fluorescence intensities at each pixel were quantitated by use of a photon-counting, microchannel plate camera. From these images, values of calcium-dependent fluorescence intensity averages in whole cells, areas of calcium release (as zones of high intracellular calcium concentrations), and ratios of fluorescence intensity in central vs. peripheral sites were determined. Stimulation by 1 nM norepinephrine caused an increase in total free intracellular calcium and an activation of intracellular calcium release sites from subsarcolemmal pools initially and later from centrally located calcium pools. Subsequent addition of 100 nM norepinephrine failed to cause significant intracellular calcium release from centrally located pools. In contrast, forskolin exposure still released high concentrations of calcium from these central pools. These results indicate that pretreatment with even a relatively small concentration of norepinephrine causes markedly decreased subsequent intracellular calcium release from centrally located sarcoplasmic reticulum because of a refractoriness of the link between receptor activation and calcium release.

Desensitization of myocardial tissue to catecholamines is a common phenomenon in heart failure that limits the compensatory adjustment by the sympathetic nervous system. Increased release of adrenergic transmitter results, which leads to further desensitization of the beta-adrenoceptors that is hypothesized to involve an internalization (1) and

*To whom reprint request should be addressed: Dr. Kent Hermsmeyer, Cardiovascular Research Lab, Chiles Research Institute, Providence Medical Center, 4805 NE Glisan Street, Portland, Oregon, OR 97213.

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phosphorylation of the receptor (2), although the cAMP second messenger system can still be directly stimulated, both in vitro (3) and in vivo (4). In addition to mechanisms located at the cell membrane leading to desensitization, there is evidence that intracellular calcium release from sarcoplasmic reticulum, upon repeated exposure to hormones acting through inositol 1,4,5-trisphosphate, might contribute to decreased hormone sensitivity (5). In order to determine whether redistribution of calcium contributes to norepinephrine (NE) desensitization, intracellular free calcium concentration, (Ca^{2+}_i), and subcellular Ca^{2+} localization were measured in cardiac muscle cells from neonatal animals, which have a high NE sensitivity probably because they have not been previously exposed to more than trace amounts of catecholamines (6).

Methods

Isolation of single ventricular myocardial cells:
Primary cultures of cardiac muscle cells were prepared from ventricles of 15-20 decapitated 0- to 3-day old Wistar-Kyoto rats as described in detail elsewhere (7). The cells were dispersed and plated at low density (100,000 cells) onto polylysine-coated glass coverslips.

Two days later, the coverslips were placed in a laminar flow chamber (6) and a single, spontaneously contracting cell was observed at 750x magnification on a Leitz-Diavert microscope. In order to load cells with fura2, 20 ul of a fura2-acetoxymethylester stock-solution (1 uM fura2-AM in DMSO containing 0.05% pluronic 127) was added to the chamber (volume 300 ul). After 5 min extraneous fura2 was washed away with ionic solutions for mammals (ISM), consisting of (mM): 143 Na^+, 4.7 K^+, 1.8 Ca^{2+}, 0.8 Mg^{2+}, 136 Cl^-, 16 HCO_3^-, 0.4 SO_4^{2-}, and 17 Hepes. Except for loading, chambers were continuously perfused with either ISM (control) or drugs in ISM. The cells were exposed for 5 min to 1 nM NE and 0-30 min later either to 100 nM NE or 1 uM forskolin (FS). Temperature during the entire procedure was kept constant at 37°C.

Fluoresence intensities were detected and digitized at 340, 360, and 380 nm excitation and 510 nm emission by a VIM microchannel plate, photon-counting camera (Photonics Microscopy Inc.), using 10 nm band filters from Corion and Oriel, as fully described elsewhere (8), with excitation filters in a moving camera which allowed for rapid switching among the 3 wavelengths. To correct for day-to-day variability of the light source (mercury lamp) and detector, fluorescence intensities were standardized on phosphor beads cemented in the chamber. At 750x magnification, resolution

was 0.3 um. Continuous recordings were made using a Sony VO 5800 videocassette recorder and, to allow for later detailed analysis, digitized images were stored on a Hewlett-Packard Vectra computer and an ISI laser disc.

Determination of Ca^{2+}_i is based on analysis of the Ca^{2+}-dependent fluorescence ratio, on a pixel/pixel basis, related to control levels (100%) and are expressed as %-change. To analyze digital photon-counted images, recordings from videotape were accumulated for 0.25 to 0.5 sec. The following equation was applied to each pixel: I=AxBxCx(P340/P380)-D, where P represents the number of photon counts at the respective wavelengths, A adjusts for day-to-day lamp and camera variability, B corrects for fura2 leaking, C was a constant scaling factor to fill the complete 8-bit digitizer and D was the pre-fura2 stray light, autofluorescence, and background noise. Images accumulated (P340/P380) for computer analysis were matched pairs from adjacent 0.5 sec intervals.

Due to the inhomogeneous distribution of Ca^{2+}_i, size and mean intensities of regions with fluorescence 2.5 times greater than the mean of the entire cell were defined and treated as hot spots (HS). In addition, mean intensities in central and peripheral regions were analyzed separately to allow for the calculation of the ratio (C/P). The peripheral boundary of the cell was defined as within 1.5 um from the edge.

Phosphor beads (with minimum fluorescence fading) were provided by Sylvania GTE Products Co., Towanda, PA. All other compounds were from Sigma. Statistical analysis was performed using paired student t-tests where $p < 0.05$ was accepted as significantly different. Results are presented as mean $\pm$ standard deviations (SD).

Results and Discussion

To study Ca^{2+} release in single, myocardial cells, we used fura2 as a sensitive indicator with high fluorescence intensity (9) to localize Ca^{2+}_i and a highly sensitive, photon-counting camera to quantitate fluorescence with 100x more sensitivity than intensified silicon target cameras. We used Ca^{2+} comparison to resting levels because various procedures for determining saturating and minimal Ca^{2+} binding to fura2 for quantitation of Ca^{2+}, (ionomycin, triton X-100, digitonin) all resulted in changes of cell shape, dye distribution, and contraction and, therefore, could not be reliably used in these cells.

Even in non-activated cells, an inhomogenous distribution of cytosolic free Ca^{2+} was prominent in all cells. These hot spots

TABLE

Ca^{2+} release changes with repeat stimulation in rat ventricular myocardial cells (means ± standard deviations)

	ti (%)	C/P	Hot spots number	%sHS	sHS (sq um)
A) (n=7)					
-Control	100	1.9±0.2	7±1	15±4	5±3
-NE (1 nM)	115±7	2.7±0.8	11±4	22±9	10±3
-subsequent NE (100 nM)	98±4	1.9±0.2	9±2	16±8	9±3
B) (n=12)					
-Control	100	2.0±0.3	4±1	13±5	5±1
-NE (1 nM)	118±7	2.3±0.3	6±5	19±3	6±0.3
-subsequent FS (1 uM)	287±31	2.6±0.2	10±5	24±6	7±0.4

(HS) were clearly present even after correction for the non-uniform fura2 concentrations by the image at 360 nm, the Ca^{2+}-independent wavelength. Non-uniform Ca^{2+} distribution and the Ca^{2+}-insensitive wavelength correction for fura2 concentraion have also recently been reported by others (10). We have arbitrarily defined HS as areas with a Ca^{2+}-dependent intensity at least 2.5 times greater than the mean intensity of the entire cell. HS presumably reflect regions of high Ca^{2+} concentration caused by release or uptake sites which maintain a steep Ca^{2+} gradient. In resting (control) cells, the area represented by HS was 15% of the whole cell (%sHS, see TABLE).

To investigate beta-adrenergic receptor stimulation of intracellular Ca^{2+} release, NE was used as a desensitizing neurotransmitter (11). The addition of 1 nM NE caused a rise in average Ca^{2+}-dependent fluorescence which peaked within 20 sec at 115% of the control (ti in TABLE). This rise is small compared to the value of 206±17% observed with 100 nM NE without any pretreatment. Stimulation of the cell with 1 nM NE also caused a rise in %sHS from 15 to 22% as well as an increase of absolute size and the number of HS; and there was an increased

C/P indicating that in addition to Ca^{2+} influx, intracellular Ca^{2+} is released from central sarcoplasmic reticulum (SR) sites (12). Combining the effects induced by 1 nM NE (protocols A and B in TABLE), all drug exposures were significantly different than controls ($p < 0.05$). If 100 nM NE was added subsequent to 1 nM pretreatment (protocol A), no significant change could be observed, whether in ti or in localization, C/P or HS. In contrast, if the cell was exposed to 1 uM forskolin instead of a second dose of NE, more pronounced Ca^{2+} increases were observed. This indicates that the beta-adrenoceptor coupled guanylate cyclase system is still responsive, able to cause release of Ca^{2+} from the central SR. Provided that intracellular Ca^{2+} release from the SR is regulated by Ca^{2+} mobilizing mechanisms, perhaps including inositol 1,4,5-trisphosphate as in other cells (5), the release of Ca^{2+} by forskolin detected in this study shows that these mechanisms between receptor activation and Ca^{2+} release probably explain the refractoriness.

In conclusion, the results reported in this study demonstrate that (a) intracellular Ca^{2+} release occurs heterogeneously, even in unstimulated cells, (b) NE releases Ca^{2+} significantly from centrally located SR, and (c) this hormone-specific release is refractory to a second exposure of NE, but (d) stimulation of the subsequent intracellular cascade process is still able to release Ca^{2+} from the same pools.

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References

1. Hertel C., Mueller F., Portenlier M., and Staehelin M. (1983) Biochem J. 216, 669-674.

2. Lefkowitz R.J., Caron M.G., and Stiles G.L. (1984) N. Engl. J. Med. 310, 1570-1579.

3. Sibley D.R., and Lefkowitz R.J. (1985) Nature (London) 317, 124-129.

4. Erne P., Lipkin D., and Maseri A. Impaired beta-adrenergic receptor and normal postreceptor responsiveness in patients with congestive heart failure, Am J Cardiol., in press.

5. Prentki M, TJ Biden, D Janjic, RF Irvine, MJ Berridge, CB Wollheim. Nature 309: 562-564 (1984)

6. Hermsmeyer K., and Robinson R. (1977) Am. J. Physiol. 233, C172-C179.

7. Marvin W. Robinson R.B., and Hermsmeyer K. (1979) Circ Res 45, 528-540.

8. Erne P., and Hermsmeyer, K. Modulation of calcium-release sites by potassium channel openers in vascular muscle. (submitted)

9. Grynkiewicz G., Poenie M., Tsien R.Y. (1985) J. Biol. Chem. 260, 3440-3449.

10. Wier W.G., Cannell M.B., Marban E., and Lederer W.J. (1987) Science 235, 325-328.

11. Hermsmeyer K., Mason R., Griffen S.H., Becker P. (1982) Circ Res 51, 532-537

12. Somlyo A.P. (1980) Handbook of Physiology, pp. 33-67, American Physiological Society, Bethesda, MD.