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Report to European Society of Cardiology – First Initiative Contact Grant

First of all I would like to thank the members of the ESC council for providing me a grant in autumn 2011. It has been essential for deeper understanding of my current subject of research, as well as for my career development. The grant was used to visit the lab of Prof. Wolfgang Dostmann at the department of pharmacology in the University of Vermont, Burlington, USA. During my stay in Prof. Dostmann's lab I had the opportunity to present data of my current project, learn new techniques, and find new directions for the project I am involved in at the moment. At the end of the visit a decision to join his lab as a postdoctoral fellow has been done.

My current research project on "Ca²⁺-handling proteins in atrial fibrillation (AF)" deals with nitric oxide (NO)-mediated regulation of Ca²⁺-handling proteins in human atrial tissue. In atrial cardiomyocytes from patients in sinus rhythm, the NO donor SNAP increases basal L-type Ca²⁺ current (I_{Ca,L}) and decreases isoprenaline-stimulated I_{Ca,L}. However, in AF-cardiomyocytes, the enhancing effect of the NO donor on I_{Ca,L} is blunted, whereas the inhibitory effect on isoprenaline-stimulated I_{Ca,L} is preserved. We have evidence that the observed NO donor effects are related to cGMP-cAMP cross-talk mediated via phosphodiesterase 2 (PDE2) and PDE3 and their respective compartmentation within the cell. For proof-of-concept we require direct spatio-temporal measurements of the cyclic nucleotides.

Though the use of FRET-based cyclic nucleotide indicators has certainly added to our knowledge about spatiotemporal regulation of cyclic nucleotides, this method has several disadvantages. Indeed, the low changes in the overall emission, as well as the low sensitivity of the technique make the detection of small fluctuations difficult (1). Thus, FRET-based indicators might not enable the tracking of small, yet physiologically relevant changes in cyclic nucleotides levels.

Few years ago a new non-FRET based cGMP biosensor named δ -FlnG has been developed and tested in Wolfgang Dostmann's laboratory and was demonstrated to have superior spectral characteristics, fast association and dissociation kinetics, allowing capture of the very rapid rise and fall in cGMP, and greatly improved environmental stability (2).

The aim of my visit was: to characterize the behaviour of FlnG-biosensor in cardiac cells. Initially we planed to purify the FlnG protein and subsequently apply it through the patch micropipette to native isolated cardiac myocytes. We used

B21(D3) strain of E.coli to overexpress the protein of interest, which contains 6xHis tag. However, we had some difficulties to purify an active protein. Therefore, we used another strategy: adenovirus-based overexpression of FlnG in the mouse cardiac cell line HL-1 (kindly provided by Dr. Claycomb) and primary rat aorta smooth muscle cells (RASMC).

RASMC were used as a control of FlnG behaviour, HL-1 cells were chosen because of the relatively easy handling and close phenotype to primary cardiac cells (2, 3). Both cell types were transfected with an adenoviral construct that carries the FlnG. Cells were incubated with it approximately 16 hours at 37°C and 5% CO₂ atmosphere before imaging.

Nikon Diaphot 200 inverted microscope was used for epi-fluorescence imaging. FlnG has a maximum absorption at 491 nm, excitation at 480 nm yielding a maximum emission at 511 nm, which is shifted when cGMP binds ($K_D \sim 170$ nM; Nausch *et al.*, 2008). MAHMA NONOate (Methylamine hexamethylene methylamine NONOate, $t_{1/2} = 63$ s at 37°C) and Spermine NONOate ($t_{1/2} = 39$ min at 37°C) have been used as NO donors. CPTIO (2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) was used as a scavenger of NO to achieve an appropriate concentration of NO, **10nM** (4). A chamber for measurement was heated constantly to 37°C.

The data were analysed using MetaMorph, Excel and GraphPadPrizm 5.0. Few areas of interest (AOIs) were selected for each FlnG-expressing cell and the mean fluorescence intensity was measured over time. The background (F_0), which was selected in cell-free areas, was subtracted from all values of the mean fluorescence intensity (F) measured for the whole experiment, giving ΔF . Normalisation of the data has been done $\Delta F/F_0$; all cells were averaged to show the mean FlnG readout of the experiment.

Results. In RASMC addition of 10 nM NO with SPERMINE/NONO increased a fluorescence for $22.5\% \pm 2.8$ ($n = 9$ cells / $N = 30$ AOIs). Similarly, in HL-1 cells during the application of 10 nM NO we observed a fluorescence increase of $26.3\% \pm 1.6$ ($n/N = 30/43$) and $19.6\% \pm 1.1$ ($n/N = 6/17$) with Spermine/NONOate and MAHMA/NONOate, respectively (**Figure 1A**). We did not perform a calibration of FlnG and $[cGMP]_i$ for HL-1 cells. However, according to published data in RASMC, 24% increase of fluorescence would correspond to increase in 1 μ M cGMP (2). The fluorescence increase during NO application was reversed by sGC inhibitor, ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, 10 μ M) (**Figure 1B**).

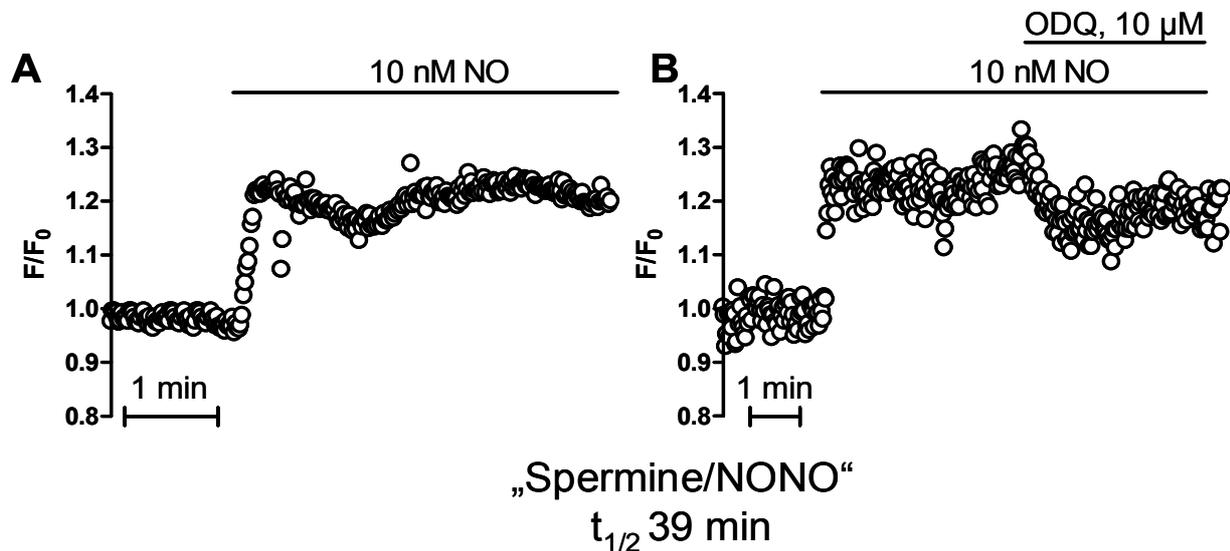


Figure 1. Temporal dynamics of cGMP in HL-1 cells. **A**, representative trace of change in the fluorescence upon addition of 10 nM NO with the Spermium NONOate donor; **B**, change in the fluorescence during addition of 10 nM NO and inhibition of sGC with ODQ (10 μ M) in the continuous presence of NO.

Such preliminary results allow us to assume that non-FRET based cGMP biosensor, FlnG, can be used for the study of spatio-temporal signalling of cGMP in cardiac cells. However, further experiments with human and mouse primary cardiac myocytes and adenoviral-based overexpression of FlnG are going to be performed in the laboratory in Dresden. We realize that the chance of success with primary cardiac cells, especially with human cells, is quite low. Therefore, modifications of FlnG might be needed to adopt the technique to our cells of interest.

Yours sincerely,
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