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A Sensitive Method for Measurements of Luminescence in Single Cells

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A method was developed to monitor changes in cytoplasmic ATP concentration in intact living insulin-producing cells. Luciferase was introduced into HIT M2.2 cells (Santerre e al. 1981, Edlund et al. 1985) by transient expression of firefly (*Photinus* pyrialis) luciferase cDNA (de Wet et al. 1987). In transfected cells, extracellular addition of luciferin increased the luminescence signal to a mixmum within 50 to 120 seconds. Addition of 0.3, 3, and 30 mM azide (NaN_3) decreased the luminescence in a dose-dependent manner; this effect was completely reversed upon withdrawal of the compound. Changes in the luminescence signal were parallelled by changes in cellular ATP, ADP, and AMP contents and in activity of the ATP-sensitive K^+channel (K_{ATP} channel).

This method may be used to monitor changes in cytoplasmic ATP in real time and thereby to assess the role of ATP in different key regulatory steps of the simulus-secretion coupling in the insulin secreting β-cell. Thus, in the future it should be possible, using imaging techniques, to monitor local changes in ATP concentration in well defined areas of the cell.

For optimization of transfection, luciferase activity was quantified in extracts from transfected cells using a liquid scintillation counter. As means of transfection, calcium phosphate precipitation, lipofectin, and electroporation were tested. In our hands, electroporation produced the highest and most reproducible expression. The choice of heterologous promotor to drive the luciferase cDNA was based on analysis of CAT reporter gene constructs driven by the rat, human insulin, SV40 and RSV promotors. This demonstrated that the relative promotor activities were approximately 80, 1, 20 and 100, respectively. We therefore used the RSV-driven luciferase construction (pRSVL).

Cells attached to coverslips were superfused in a custom built open chamber with a flow rate of 0.1 ml/min. The chamber was mounted on an inverted microscope (Zeiss Axiovert 135TV, Zeiss, Germany) equipped with a photon-counting photometer tube, chilled to between -30 and -25°C with a custom made water cooled peltier element. Lenses were Zeiss Achrostigmat 40x/1.30 Oil and, for imaging, Zeiss Fluar 40x/1.30 Oil. Data acquisition was performed with hardware from Spex Industries (Edison, NJ). Integration time was 5 seconds per time point and background (dark) signal was in average 2.5 counts per second (cps). Acquisition of images was done with a cooled CCD (Charge Coupled Device) camera (CH50 with KAF 1400, Photometrics Ltd., Tucson, AZ) connected to an imaging system (Inovision Corp., Durham, NC). Integration time used for images was 300 seconds. Temperature during experiments was 33°C. In order to quantify the effect of azide on luminescence, linear functions were curve fitted to the trace before, during, and after the lowering effect of the metabolic inhibitor was detected. Curve fitting was necessary because of the decay of luminescence and the signal to noise ratio. As shown in Figure 1E, we denote the endpoints of the fitted functions S_1, S_2, S_3, and S_4. These endpoints were corrected for background (dark counts) in respective experiment. The effect on luminescence (Δ-luminescence) was then calculated as $(S_2\text{-}S_1)/S_1$ (Fig. 1F), and the degree of recovery after withdrawal of azide was calculated as $\{(S_4\text{-}S_3)/S_4\}/\{(S_2/S_1)/S_1\}$ (Fig. 1G). Only experiments in which the background was lower than 4 cps were included in the calculations.

To investigate the correlation between changes in ATP concentration and changes in lumi-

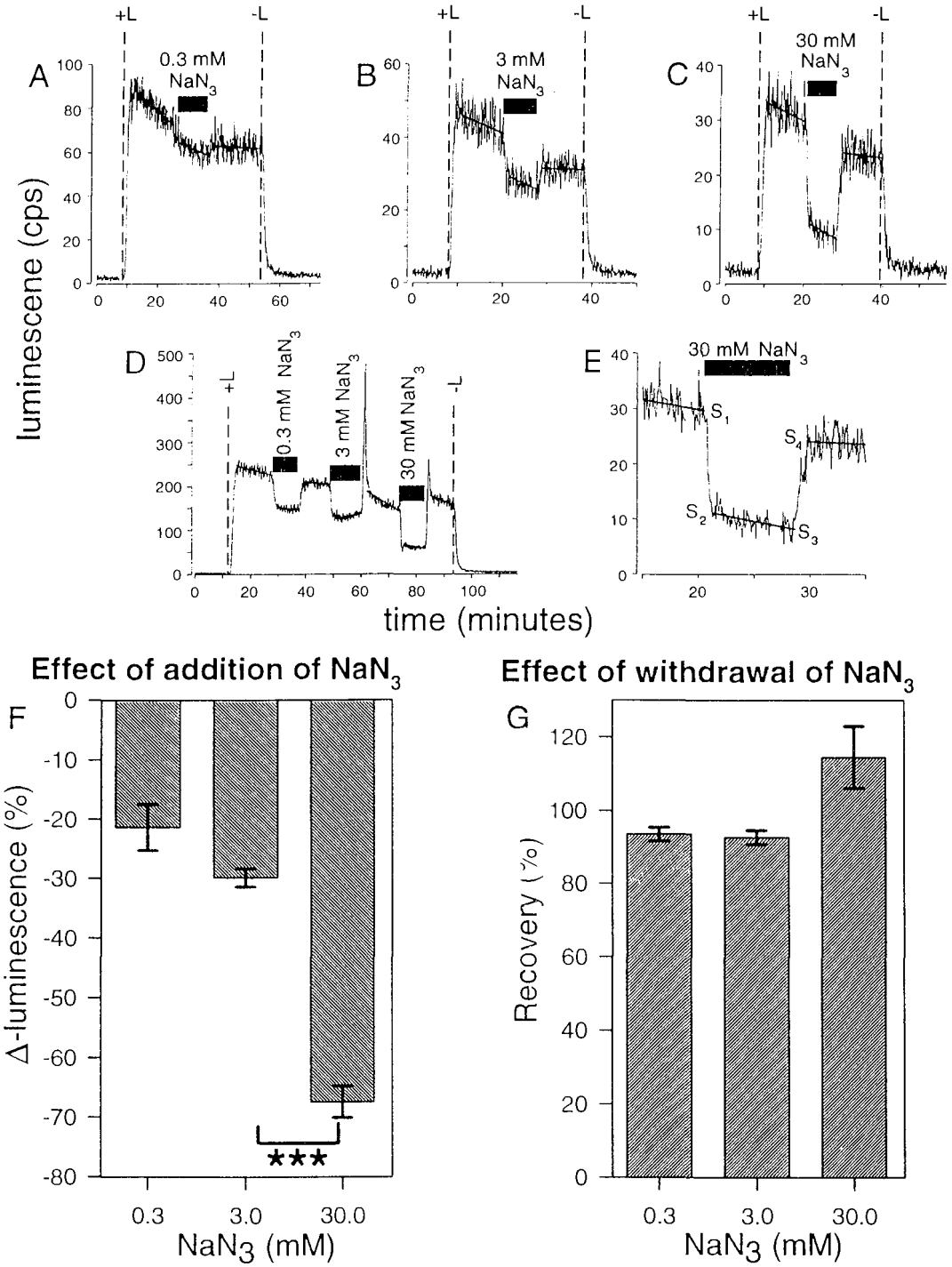

Fig. 1. Luminescence detected from groups of luciferase expressing HIT M2.2 cells (20-30 in the measuring field), during exposure to 0.1 mM D-luciferin added to theextracellular medium. Addition of azide (0.3, 3, and 30 mM), as indicated in the figure, was accompanied with a decrease in luminescence. The linear fitted functions to the traces before, during and after azide exposure are included in the figure, was accompanied with a decrease in luminescence. The linear fitted functions ot the traces before, during and after azide exposure are included in the figure. Due to light artifacts short segments of the trace were excluded when changing perifusion buffers. **A-D:**"+L" denotes addition of D-luciferin and "-L" denotes withdrawal of D-luciferin. **E:** Part of Figure C showing the endpoints (S_1, S_2, S_3 and S_4) of the linear functions fitted to the luminescence traces, before, during and after the response to azide. **F:** The luminescence effect in response to azide (Δ-luminescence) given in percent (%) and calculated as $(S_2-S_1)/S_1$. Effects of 0.3, 3, and 30 mM azide are plotted as mean ±SEM(n=5, 10, and 6, respectively). The effect at 30 mM azide is significantly lower than at 3 mM of the metabolic inhibitor (***p<0.001), as calculated with Student's unpaired t-test. **G:** The recovery of luminescence afte withdrawal of azide, given in percent (%) and calculated as $\{(S_4-S_3)/S_4\}/\{(S_2-S_1)\}$.

nescence, we exposed cells to 0.3, 3, and 30 mM sodium azide (NaN_3), a mitochondrial electron transport inhibitor of cytochrome a_3. We then compared theresponses in luminescence with two other methods of ATP detection: K_{ATP} channel activity and total ATP content.

The luminescence from 10-40 transfected cells, in the same field of view, was measured in the microscopic system. Addition of 0.1 mM D-luciferin to the extracellular medium resulted in an increase in signal, which reached a maximal value within 50 to 120 s. Thereafter luminescence decayed, with slightly varying kinetics in the different experiments. In response to addition of azide, a decrease of the luminescence signal was recorded and could be reversed by withdrawal of the inhibitor. The relative decrease of luminescence (Δ-luminescence) depended on azide concentration (Fig. 1). Luminescence from a field of cells, as well as distribution in single cells, was monitored with image acquisition by a CCD camera. The fraction of cells expressing luciferase was small at the time of the experiment and the degree of expression was quite variable (Fig. 2). However, luminescence was apparently evenly distributed within each cell.

K_{ATP} channel activity is regulated by cytosolic ATP (Cook and Hales 1984, Ashcroft et al. 1984) and this channel will thus serve as a biosensor of the ATP concentration in the submembrane space. We used the patch-clamp technique to study changes in membrane potential and K_{ATP} channel activity in response to azide. The electrophysiological experiments show a similar temporal and quantitative response to azide as the luminescence measurements. Azide (3 mM) hyperpolarizes the cell within 30 s from the plaeau potential of -50 mV to approximately -80 mV. Withdrawal of the compound induces depolarizationof the cell and concomitant reappearance of overshooting action potentials. In parallel experiments, we recorded whole-cell K_{ATP}-currents by alterna-tively stepping the potential to -60 and -80 mV, repeatedly, from a holding potential of -70 mV. Following 3 mM azide, an aproximately 5-fold increase in channel activity was observed. In order to more directly study the effects of azide on the K_{ATP}-channel current, we recorded single-channel activity using the cell-attached patch configuration. In the presence of 3 mM glucose, little channel activity was observed. The number of channel openings dramatically increased upon exposure to 3 mM azide. In the presence of 30 mM azide, the channel activity was further increased and the opeings were more prolonged. These effects were fully reversed by withdrawal of the metabolic inhibitor.

The K_{ATP} channel activity is inhibited by ATP but stimulated by ADP. In fact the ATP/ADP ratio, rather than the ATP concentration *per se*, is considered the actual parameter controlling channel activity (Ashcroft and Rorsman 1989). In order to further verify that the actual exposure to azide indeed decreased both the ATP concentration and the ATP/ADP ratio, we measured the total content of ATP, ADP, and AMP in our cells, using HPLC analysis. The ATP content, following addition of azide, was decreased in a dose-dependent manner (significant changes between

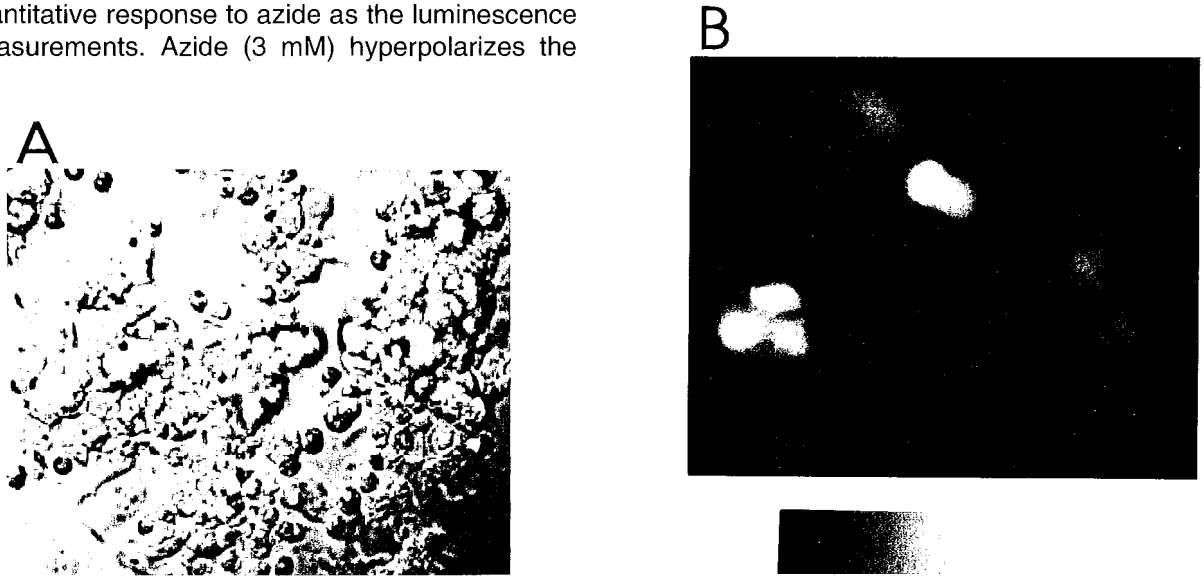

Fig. 2. A: Luciferase expressing HIT M2.2 cells visualized with DIC (differential interference contrast).
B: The same field (and the same focus) as in A shwing luminescence when the cells are exposed to 0.1 mM D-luciferin.

0.3 and 3 mM azide as well as between 3 and 30 mM of the metabolic inhibitor). Also the ATP/ADP ratio was decreased (significant between 0.3 and 3 mM as well as between 3 and 30 mM azide), as well as the total adenine nucleotide content (significant between 3 and 30 mM azide).

REFERENCES

Santerre RF, RA Cook, RMD Crisel, JD Sharp, RJ Schmidt, DC Williams, CP Wilson. 1981. Proc. Natl. Acad. Sci. USA **78:** 4339-4343.

Edlund T, MD Walker, PJ Barr, WJ Rutter. 1985. Science **230:** 912-916.

de Wet J, KV Wood, M DeLuca, DR Helinski, S Subramani. 1987. Mol. Cell. Biol. **7:** 725-737.

Cook DL, CN Hales. 1984. Nature **311:** 271-273.

Ashcroft FM, DE Harrison, SJH Ashcroft. 1984. Nature **312:** 446-448.

Ashcroft FM, P Rorsman. 1989. Prog. Biophys. Mol. Biol. **54:** 87-143.