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RESEARCH ARTICLE

ATP distribution and localization of mitochondria in *Suberites domuncula* (Olivi 1792) tissue

Lada Lukić-Bilela[1,2,*], Sanja Perović-Ottstadt[3], Stefan Walenta[4], Filipe Natalio[2], Bruna Pleše[2,5], Thorben Link[2] and Werner E. G. Müller[2]

[1]Laboratory for Molecular Genetics of Natural Resources, Institute for Genetic Engineering and Biotechnology, Gajev trg 4, 71 000 Sarajevo, Bosnia and Herzegovina, [2]Institute for Physiological Chemistry, Department for Applied Molecular Biology, Johannes Gutenberg-University Medical Center, Duesbergweg 6, D-55099 Mainz, Germany, [3]BIOTECmarin GmbH, Duesbergweg 6, 55099 Mainz, Germany, [4]Institute of Physiology and Pathophysiology, Johannes Gutenberg-University Medical Center, Duesbergweg 6, D-55099 Mainz, Germany and [5]Department of Molecular Biology, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia

*Author for correspondence (lada.lukic@ingeb.ba)

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SUMMARY

The metabolic energy state of sponge tissue *in vivo* is largely unknown. Quantitative bioluminescence-based imaging was used to analyze the ATP distribution of *Suberites domuncula* (Olivi 1792) tissue, in relation to differences between the cortex and the medulla. This method provides a quantitative picture of the ATP distribution closely reflecting the *in vivo* situation. The obtained data suggest that the highest ATP content occurs around channels in the sponge medulla. HPLC reverse-phase C-18, used for measurement of ATP content, established a value of 1.62 μmol ATP g^{-1} dry mass in sponge medulla, as opposed to 0.04 μmol ATP g^{-1} dry mass in the cortex, thus indicating a specific and defined energy distribution. These results correlate with the mitochondria localization, determined using primary antibodies against cytochrome oxidase *c* subunit 1 (COX1) (immunostaining), as well as with the distribution of arginine kinase (AK), essential for cellular energy metabolism (*in situ* hybridization with AK from *S. domuncula*; *SDAK*), in sponge sections. The highest energy consumption seemed to occur in choanocytes, the cells that drive the water through the channel system of the sponge body. Taken together, these results showed that the majority of energetic metabolism in *S. domuncula* occurs in the medulla, in the proximity of aqueous channels.

Key words: ATP distribution, mitochondria, imaging bioluminescence, HPLC, Porifera, *Suberites domuncula*.

INTRODUCTION

The number of mitochondria, the main producers of energy required for cellular function, varies from 20 to 2500 per cell (Pike and Brown, 1984). As in most eukaryotic cells, the main source of adenosine triphosphate (ATP) in sponges is generated by mitochondrial oxidative phosphorylation (Sona et al., 2004). The highest energy consumption should be localized in choanocytes, which are the 'motor' cells that drive the water through the aqueous channel system (Perović-Ottstadt et al., 2005). The choanocytes of sponges are structurally and functionally identical to choanoflagellates, which facilitated speculations about the evolutionary relationship between the two groups (Barnes, 1987). The crucial difference between sponges and choanoflagellates is the ability of sponges to retain and digest the food particles in the extensive channel system, before the final filtration through the collar slits of choanocytes (Kilian, 1952; Simpson, 1984). Compared with other filter-feeding invertebrates, sponges have low water pumping capacity, but a surprisingly high retention efficiency for small particles >0.1 μm (Thomassen and Riisgard, 1995).

The enzymes that catalyze reversible transfer of a phosphoryl group between a phosphorylated guanidine (phosphagen) compound and adenosine diphosphate (ADP) are phosphagen kinases (Suzuki et al., 1997). These enzymes play a central role in cellular energy metabolism by temporary buffering of ATP levels in cells with fluctuating energy requirements (muscle, nerves, etc.) and by shuttling the energy between different cellular compartments (Wallimann et al., 1992). Arginine kinase (AK), as a member of the phosphagen kinases in invertebrates (Kenyon and Reed, 1983; Strong and Ellington, 1993), is highly expressed in energetically demanding reactions such as the formation of the siliceous skeleton, which has been demonstrated in *Suberites domuncula* (Perović-Ottstadt et al., 2005).

In the present study we used a cDNA portion of AK as a probe for *in situ* localization studies and immunohistological analysis to determine the bulk of ATP production in the sections of sponge tissue. To further describe the energy status we analyzed the ATP distribution in two different parts of sponge tissue (medulla and cortex) by imaging bioluminescence. This technique has been successfully applied in the past for quantitative localization of metabolites in cryosections of various biological tissues (Walenta et al., 1990; Müller-Klieser and Walenta, 1993; Borisjuk et al., 1993; Levin et al., 1999; Walenta et al., 2002; Borisjuk et al., 2002a; Borisjuk et al., 2002b; Walenta et al., 2004). The bioluminescence-based imaging technique results in an image of the ATP distribution that closely reflects the situation *in vivo*. Because the calibration procedure depends on the specific structure of higher animal or human tissues, the method was used semi-quantitatively. In addition,

the ATP content in both the medulla and the cortex was determined by HPLC.

MATERIALS AND METHODS

Chemicals and enzymes

The sources of chemicals and enzymes for *in situ* hybridization and for ATP-measurement were given previously (Perović et al., 2003; Müller-Klieser and Walenta, 1993). The primary antibodies against oxidative complex IV [cytochrome *c* oxidase subunit 1 (COX1); A6403] were purchased from Invitrogen (Carlsbad, CA, USA) and the secondary antibodies (Cy3-conjugated goat anti-mouse IgG; 115-165-062) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Sponges

Live specimens of *Suberites domuncula* (Olivi 1792) (Demospongiae, Tetractinomorpha, Hadromerida, Suberitidae) were collected in the Northern Adriatic Sea near Rovinj, Croatia, at depths of 33 m. The temperature was maintained at 16°C during transfer of the sponges to the laboratory. They were kept in an aquarium in natural seawater at 17°C for 5–7 days before utilization.

In situ localization studies and immunohistology

The method applied was based on the procedure described by Polak and McGree (Polak and McGree, 1998) with modifications (Perović et al., 2003). Cryosections through sponge tissue (10 μm thick) were cut using a microtome, air-dried for 30 min at room temperature, treated with 1 μg ml^{-1} proteinase K (30 min at room temperature) and subsequently fixed again for 10 min with 4% (w/v) paraformaldehyde solution [4% (w/v)]. To remove the typical sponge color, the sections were treated with different concentrations of ethanol. After rehydration, sections were hybridized overnight at 40°C [23 sodium chloride-sodium citrate buffer (SSC), 50% formamide] with DIG-labeled probe, a 250 nt *Suberites domuncula* arginine kinase (*SDAK*) cDNA portion (Perovic-Ottstadt et al., 2005). The sections were washed at 45°C in 23 SSC and 0.23 SSC. After blocking in PBS-T solution [2% blocking reagents for nucleic acid (Roche, Manheim, Germany) prepared in 13 PBS, containing Tween 200.1% (v/v)] for 15 min at room temperature the sections were incubated with the primary antibody (A6403, Invitrogen; 1:200 dilution in 1% blocking solution) against oxidative complex IV (COX1) for 2 h at 37°C. Subsequently, the sections were washed twice for 5 min with PBS-T 2 mg ml^{-1} bovine serum albumin (BSA) at room temperature. Afterwards, the cryosections were incubated with Cy3-conjugated goat anti-mouse IgG secondary antibodies (1:200 dilution prepared in 1% blocking solution) and anti-DIG Fab fragments (Roche) conjugated with alkaline phosphatase (AP; 1:100 dilution) for 1 h at 37°C in a humid chamber. The dye reagent NBT/X-Phosphate was used for visualization of the *in situ* signals. After the reaction, the sections were stained with 49,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich, Steinheim, Germany) for 30 min at room temperature in 23 SSC. Finally, the sections were washed in 13 PBS, embedded in fluorescent mounting medium (Dako, Hamburg, Germany) and analyzed with a microscope.

DNA oligonucleotide probes were constructed as described previously (Perovic-Ottstadt et al., 2005). Antisense and sense ssDNA DIG-labeled probes were synthesized by PCR using the PCR-DIG-Probe-Synthesis Kit (Roche) according to the manufacturer's protocol. Sense probe was used in parallel as a negative control in the experiment. The microscopical inspections and immunofluorescence analysis were done with an Olympus AHBT3 light microscope (Olympus, Hamburg, Germany) with AH3-RFC reflected light fluorescence attachment at excitation/emission wavelengths of 394/450 nm (filter U; DAPI; blue) and 550/570 nm (filter G; Cy3; red). The images were recorded with a ColorView12 camera (Soft Imaging System, Munster, Germany) and controlled by analySIS®3.0 (Olympus).

Imaging of local ATP content

An imaging technique developed for metabolic mapping in rapidly frozen human tissues was used to obtain the local ATP distribution in sponge tissue. This technique is based on highly specific bioluminescence reactions initiated in cryosections. Here, ATP was imaged using the bioluminescence system of fireflies, consisting basically of firefly luciferase and luciferin. The intensity of the light emission is proportional to the tissue content of ATP. For further details see Müller-Klieser and Walenta (Müller-Klieser and Walenta, 1993) and Walenta et al. (Walenta et al., 2002).

Tissue preparation

A piece of the sponge *S. domuncula* was cut and immediately frozen at –80°C. The frozen sponge piece was embedded in Tissue-Tek (Slee, Mainz, Germany). Serial sections, 20 μm in thickness, were made on a cryostat (Slee) at –25°C and mounted on slides under cover glasses (18×18 mm, 0.13 mm thick). To block any intrinsic enzymatic activity, sections were subjected to instant heat fixation on a heating plate at 100°C for 10 min and were subsequently stored at –20°C until further use.

Metabolic imaging

For bioluminescence imaging, cover glasses with adhered cryosections of the samples were laid upside-down on a metal slide with a casting mold. The ground of the mold is made of glass, allowing light transmission. The mold was filled with an enzyme solution containing firefly luciferase and luciferin. The mold carrying the cover glass was transferred instantaneously to a thermostated reaction chamber on the stage of an appropriate microscope (Axiophot, Zeiss, Oberkochen, Germany). After 10 s incubation time at 20°C, bioluminescence was registered for a well-defined time interval. Emission of light was detected with a 16 bit CCD camera coupled to an imaging photon counting system (Hamamatsu, Herrsching, Germany) that was connected to the microscope. The whole assembly was located within a light-tight black box to prevent registration of background photons of the surrounding environment. Subsequently, without removing the mold, a transmitted light image of the section was made using the same camera system.

Image analysis

The resulting digital bioluminescence and transmitted light images of the sponge sections were analyzed using Wasabi imaging software (Hamamatsu). The light intensities were color coded, where dark blue represents the lowest and light red represents the highest ATP content. The corresponding transmitted light images allowed identification of the cortical and medullar parts of the sponge.

HPLC

The ATP content in sponge samples was determined quantitatively using HPLC. For this purpose, cortical or medullar parts of the sponge were analyzed separately by carefully sectioning the sponge tissue. The frozen sponge tissue was frozen in liquid nitrogen, homogenized using a mortar and pestle and lyophilized

at –40°C, 0.01×10^{-3} mPa for 72 h (Secfroid; Aclens, Switzerland). Dried samples were stored at –20°C until further use. Subsequently, 50 mg each of dry tissue from the medulla and cortex were separately extracted with 500 µl of 5% (v/v) perchloric acid solution [5% (v/v)], sonificated three times for 10 s and centrifuged for 10 min at 15,700 ***g***. After adjustment of pH with KOH (2 mol l^{-1}) to neutral pH (7.0), the supernatant was collected and stored at –20°C until further use. Neutralized medulla and cortex extracts were separately injected (50 µl) into the HPLC system (consisting of the BioRad 2700 solvent delivery system, AS-100 HRLC automatic sampling system and 1801 UV Monitor; BioRad, Hercules, CA, USA), mounted with a reversed phase Superspher-100 RP-18 column (250×4 mm, 5 µm particle size; Knauer, Berlin, Germany). Samples were eluted with solution containing NH_4HPO_4 (50 mmol l^{-1}), TBA (10 mmol l^{-1}) and acetonitrile [11.5% (v/v)], pH 6.3–6.4, in pure distillated water for HPLC analysis. The flow rate was kept constant at 0.8 ml min^{-1}, measurements were performed at room temperature and ATP was detected by changes in absorbance at a wavelength of 254 nm and a precise retention time determined previously by using ATP commercially available standards. Quantification of ATP was determined based on a series of elution profiles performed using ATP standards and their respective calibration curves. All chemicals were purchased from Sigma or Roth (Roth, Karlsruhe, Germany).

RESULTS

Co-localization of mitochondria and AK in *S. domuncula*

Phosphagen kinases (creatine and AK) play a central role in energy transactions in cells displaying high and variable rates of ATP turnover. We found that the expression of mRNA for AK in *S. domuncula* was related to the immunostaining of mitochondria. Co-localization studies of AK (Fig. 1A) and mitochondria (Fig. 1B) were performed. The cryosections were hybridized with the labelled probe for AK (*SDAK*) and incubated with the primary antibodies against oxidative complex IV (COX1). In the sections of *S. domuncula*, the expression level of AK (Fig. 1A) was elevated around the sponge channels. In addition, cells close to the sponge channels had higher numbers of mitochondria compared with cells distant to the channels (Fig. 1B, Fig. 2A).

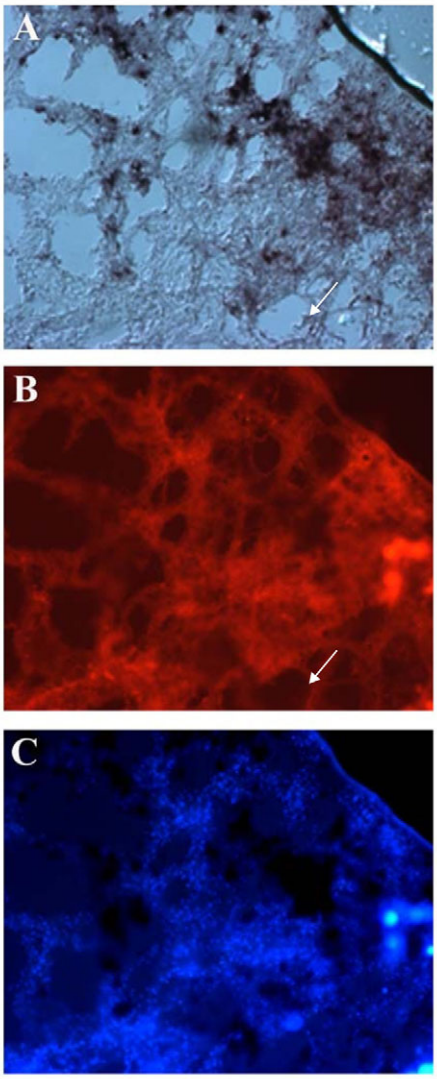

Fig. 1. Co-localization of arginine kinase and mitochondria in *Suberites domuncula*. (A) *In situ* localization of the enzyme arginine kinase detected using *SDAK* as a probe. Hybridized cells were stained with brown/black deposits. Sponge channels where expression level of arginine kinase was elevated are marked with arrow. (B) Immunostaining of *S. domuncula* cryosections, as described in the Materials and methods. The frozen sections were incubated with antibodies against cytochrome *c* oxidase (COX1). Cells close to the sponge channels had higher numbers of mitochondria compared with cells distant to the channels. (C) The slide was additionally stained with DAPI. ×200 magnification.

Distribution of mitochondria

Immunostaining of cryosections through tissue of *S. domuncula* is presented in Fig. 2A. All cryosections crossed two areas of the sponge body: the margin (cortex) and the interior (medulla). Immunostaining of the whole section showed that the majority of mitochondria were localized in the medulla region. The cells rich with mitochondria were localized around the channels (Fig. 2A).

The images of cells with mitochondria (immunostaining with COX1) were recorded as shown in Fig. 3A. Parallel (control) sections were stained with DAPI (Fig. 3B) and inspected by light microscopy (Nomarski image, Fig. 3C). The mitochondria were counted and the mean number was calculated. On the basis of our results we can postulate that *S. domuncula* cells of have approximately 15–20 mitochondria per cell, which presumably correlates with the energy requirement.

Distribution of ATP content by means of quantitative bioluminescence

To assess the ATP distribution *in vivo*, metabolic imaging was performed on snap-frozen tissue. As shown in Fig. 4, the ATP content was relatively high close to the channels and decreased toward the cortex. This correlates with our finding of a high mitochondrial density in cells that encircled the aqueous channels in the medulla. However, studies must be performed on larger groups of sponges to obtain reliable *in vivo* results with quantitative bioluminescence.

ATP measurement with HPLC

ATP concentrations in the medulla and the cortex were measured using HPLC.

Suberites domuncula medulla contained 1.62 µmol ATP g^{-1} dry mass, whereas the cortex contained only 0.04 µmol ATP g^{-1} dry mass (Fig. 5), i.e. there was a remarkably large difference in the ATP concentration (~40×) between the medulla and the cortex,

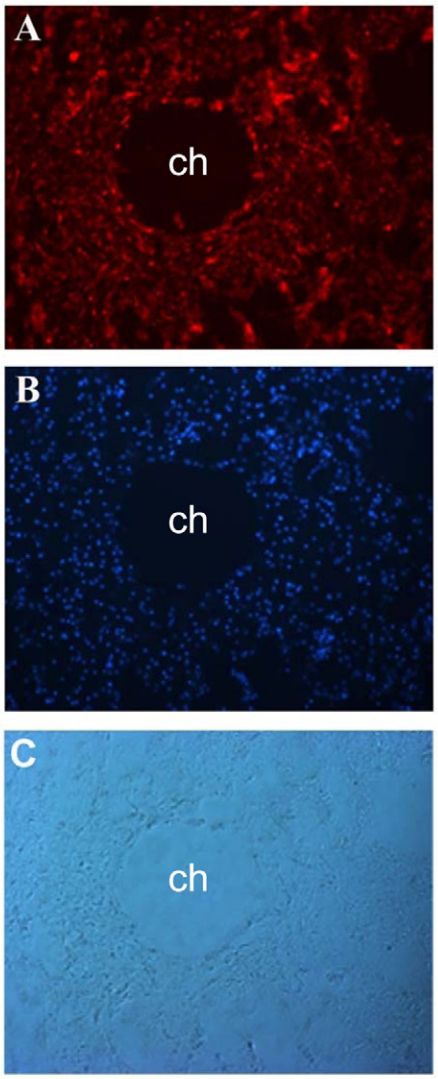

Fig. 2. Distribution of the mitochondria around the channel in *S. domuncula*. (A) Immunofluorescence microscopy of the sponge sections, which were incubated with COX1. A visible channel is marked (ch). The cells rich with mitochondria were localized around the channels. (B) The slide was additionally stained with DAPI; (C) parallel sections were inspected by light microscopy (Nomarski image). ×200 magnification.

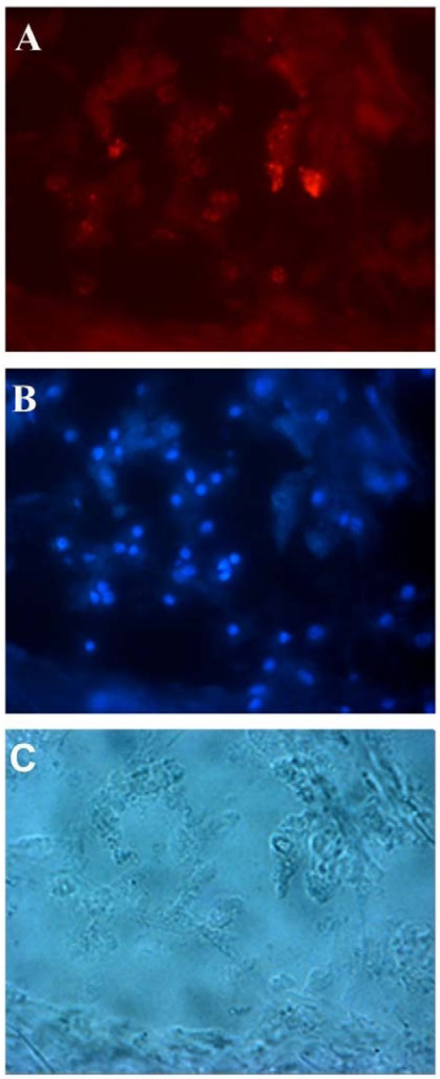

Fig. 3. Distribution of mitochondria in *S. domuncula*. The sponge sections were incubated with (A) primary antibodies against oxidative complex IV (COX1) and (B) the DNA was stained with DAPI. (C) The cells inspected are shown by Nomarski phase contrast interference optics. Channels are marked with arrows. ×1000 magnification.

suggesting different metabolic activity in different parts of the sponge. This corresponds to the ATP distributions obtained by imaging bioluminescence (Fig. 4).

DISCUSSION

Adenine nucleotides play a key role in energy metabolism in the cell, and their levels generally correlate with maximum rates of energy turnover. The role of phosphagen in the muscles is to provide a transient buffer for the concentration of ATP, thus the lowest contents of ATP and the lowest ATP/AMP concentration ratios are found in the muscles with very low energy expenditure during contraction (Beis and Newsholme, 1975).

Porifera, as the evolutionary oldest metazoan phylum, utilize AK, the ancestral phosphagen kinase for all other kinases in higher taxa. However, sponges also possess a creatine phosphate/creatine kinase system (CP/CK) (Ellington, 2000; Sona et al., 2004). The presence of both arginine phosphate (AP)/AK and CP/CK systems is a characteristic of lower chordates and deuterostome invertebrates, whereas CP/CK is the only phosphagen system found in the vertebrates. Ecdysozoans, which contain the arthropods and nematodes as the major groups, depend exclusively on the AP/AK system (Elington, 2001).

The CK/phosphocreatine (PCr) system evolved in the earliest animals to facilitate energy transport in flagellated cells. The CK lineage evolved from an AK-like ancestor *via* gene duplication and a divergence event (Ellington, 2001). The system of CK isoforms [mitochondrial (MtCK), flagellar (FlgCK) and cytoplasmic (CytCK)] evolved early, whereas MtCK, protoflagellar (protoflg)CK and FlgCK, being present in the most ancient metazoan groups (Suzuki and Ellington, 2008), were recently confirmed in demosponges and hexactinellid sponges (Bertin et al., 2007). Certainly, the primary function of the CK/PCr system in these

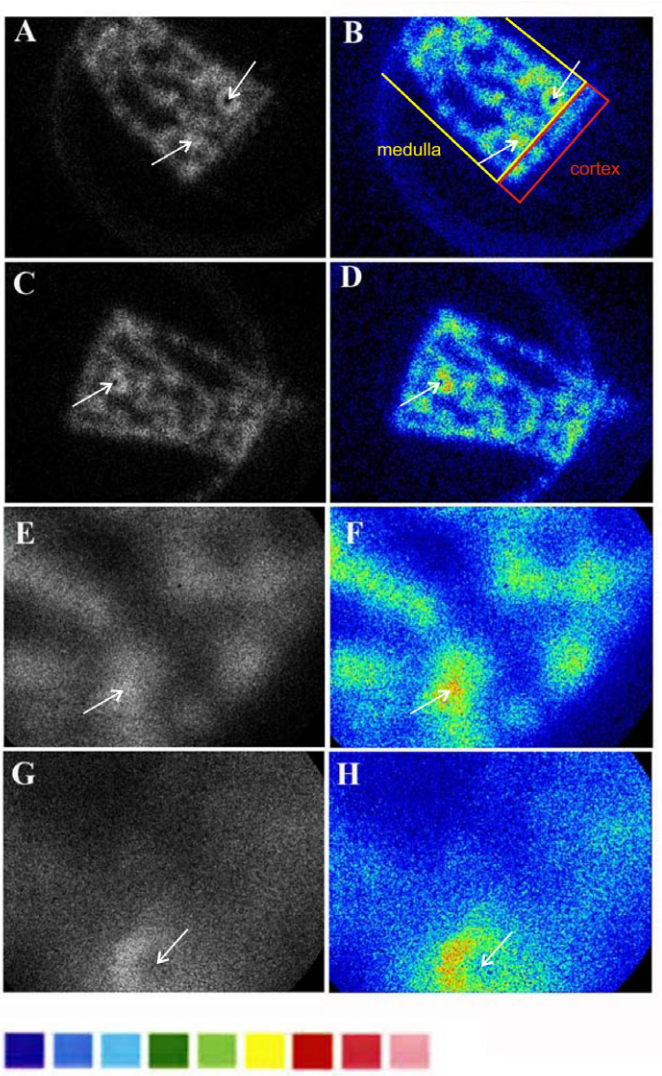

Fig. 4. Distribution of ATP content in *S. domuncula*. To assess the ATP distribution *in vivo*, metabolic imaging was performed on snap-frozen *S. domuncula* tissue (A,C,E,G). Local ATP content is color-coded, where dark blue represents the lowest and light red the highest concentration of ATP (B,D,F,H). The location of the medulla and the cortex is marked in B. ATP content was relatively high close to the channels (marked with arrow) and decreased towards the cortex. Magnification: (A–D) ×1.25, (E,F) ×5, (G,H) ×20.

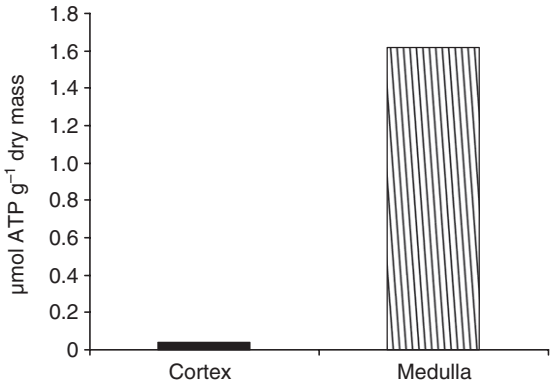

Fig. 5. ATP content in *S. domuncula*. ATP concentrations in the medulla and the cortex were measured using HPLC. The medulla contained 1.62 µmol ATP g^{-1} dry mass whereas the cortex contained only 0.04 µmol ATP g^{-1} dry mass. Such a pronounced difference in the ATP concentration corresponds to the ATP distributions obtained by imaging bioluminescence.

primitive animals is to facilitate energy transport in highly polarized cells, such as spermatozoa and choanocytes. Choanocytes are permanently in need of ATP because of flagellar beating, which is highly dependent on energy transport *via* CK or AK systems. The AK reaction (and likely CK reaction) functions as a spatial ATP buffering system and is present in other polarized cells such as spermatozoa (Tombes et al., 1985). The presence of MtCK and FlgCK in primitive type sperm was described by Tombes and Shapiro (Tombes and Shapiro, 1989) whereas the potential roles of MtCK and protoflgCK in mediation of the spatial ATP buffering energy transport pathway were proposed by Suzuki and Ellington (Suzuki and Ellington, 2008). The spatial ATP buffering by the CK/PCr system in spermatozoa and sponge choanocytes confirms that the sponge spermatozoa develop from choanocytes during gametogenesis (Kaye and Reiswig, 1991). Furthermore, because of an extreme polarization of the ATP source (mitochondria in the sperm mid-piece) and the ATP sink (dynein ATPases in the flagellum), the CK/PCr system plays a crucial role in sperm motility, creating reaction–diffusion constraints (Suzuki and Ellington, 2008).

The finding of three distinct AK genes in choanoflagellates, where two of these AKs display extensive similarity to both CKs and an AK in sponges, suggest that the initial gene duplication event occurred before the divergence of the choanoflagellates and animal lineages (Conejo et al., 2008). The choanoflagellate mitochondrial genome is more protozoan-like, with long intergenic regions; for example, in *Monosiga brevicolis*, intergenic regions constitute more than half (53%) of the mitochondrial genome (Burger et al., 2003). Codon usage and the intergenic region architecture in demospongian mitochondrial genome (*S. domuncula*) are undoubtedly more similar to choanoflagellate *M. brevicollis* than to Placozoa (*Trichoplax adhaerens*) or Cnidaria (*Metridium senile*) (Lukić-Bilela et al., 2008).

In Demospongiae, the formation of the siliceous skeleton, which is composed of spicules, is an energetically expensive reaction. AK is the crucial enzyme in energy-demanding reactions of spicule formation in primmorphs (Perović-Ottstadt et al., 2005). The phosphagen kinases are localized at the sites of ATP synthesis and hydrolysis, enabling a tuned regulation of the intracellular phosphate concentration (Ellington, 2001). Co-localization of AK and mitochondria in sponge medulla cryosections confirm the presence of phosphagen kinases in cells displaying high and variable rates of ATP turnover and/or cells in which there is a large separation between the ATP source (mitochondria) and the ATP sink (distal ATPases).

Cells with high energy demands contain the most of the energy-generating mitochondria. Brain cells, skeletal and heart muscle, and the eye contain the highest number of mitochondria (as many as 10,000 per cell) whereas the skin cells, which do not require as much energy, contain only a few hundred mitochondria.

In this study, we showed that the number of mitochondria per cell (15–20) in *S. domuncula* also correlates with the sponge's energetic requirements. Most mitochondria were localized in the medulla region, around the channels, and the ATP measurements using HPLC, quantitative bioluminescence and single photon imaging verified our results obtained with immunostaining. Our analyses demonstrate that

the majority of ATP is situated around the channels in the medulla. Aqueous channels are sheathed with choanocytes, the 'collar cells' capable of creating a water flow across the microvilli, acquired for the filtration of nutrients. The higher density of mitochondria and elevated ATP levels surrounding the water channels are likely a manifestation of the presence of choanocytes. These cells continuously drive water currents *via* the beating of a single flagellum, requiring a continuous and high recycling of ATP. Localization of mitochondria aggregations around the sponge channels confirmed our finding that the channel systems in medulla were the area where the majority of energetic metabolism was taking place.

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