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Muscle Contraction

Photocontrol of Contracting Muscle Fibers**

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The light-controlled inhibition of physiologically relevant protein–protein interactions by appropriate photoresponsive ligands in living cells or small organs (skeletal muscle fibers, vessels) could make it possible to investigate signaling pathways under high spatiotemporal control. Recently, we have reported a cyclic peptide that mimics the β-finger motif in neural NO synthase (nNOS) which is crucial for binding of nNOS to α-1-syntrophin.[1] When a photoswitchable unit is embedded into this peptide, the binding can be controlled in vitro simply by light. In skeletal muscle the extended PDZ domain of nNOS interacts with the PDZ domain of α-1-syntrophin to recruit nNOS to the dystrophin-associated protein complex in the plasma membrane, thus coupling the production of the second messenger nitric oxide (NO) to muscle contraction.[2] Loss of sarcolemmal nNOS is known to result in functional ischemia during muscle contraction, which is commonly observed in muscle diseases as Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD).[3] Herein we show that the photoswitchable peptide ligand **1** (Figure 1) is able to translocate into cells, is sufficiently stable towards intracellular conditions, and can be used in vivo to photocontrol contracting muscle fibers.

The recently described nNOS-derived, photoswitchable peptide ligand **1** of α-1-syntrophin contains the azobenzene-ω-amino acid 3-((4′-aminomethyl)phenylazo)benzoic acid (3,4′-AMPB),[4] which in its *trans* form led to a ligand that showed no affinity to the PDZ domain of α-1-syntrophin while photoisomerization to the *cis* form resulted in a remarkable affinity of the peptide ($K_D = 10.6\ \mu M$).[1a] The finding has addressed the question of the applicability of the light-controlled ligand under physiological conditions to investigate the native interference in living skeletal muscles. Light-directed binding of the *cis* form of the photoswitchable ligand **1** to syntrophin was expected to inhibit the native syntrophin–nNOS interaction in the skeletal muscle followed by the dislocation of nNOS from the sarcolemma which may result in reduced NO release from skeletal muscle cells and thus in light-controlled muscle contraction.

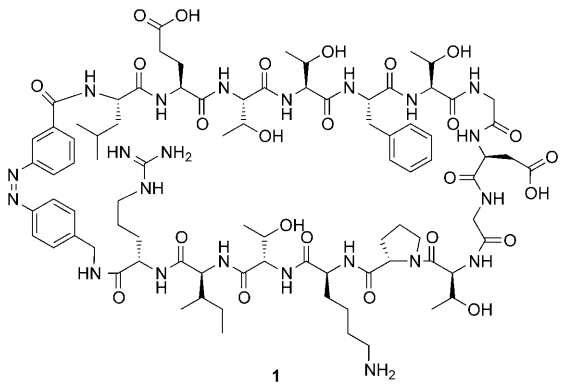

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Figure 1. Structure of the *cis* form of the photoswitchable peptide ligand related to the β-finger peptide of nNOS.

Azobenzene units have been used extensively for the photomodulation of biomolecules (peptides,[5] proteins,[6] and nucleic acids[7]) and biological processes in vitro and in vivo (as in ion channels[8]). The feasibility of using azobenzene systems in living organisms to photocontrol biological events has been confirmed by the in vivo imaging of the isomerization process in zebrafish.[9] An intrinsic hindrance for applications of azobenzene in living cells derives from its susceptibility to reduction. The azo unit may be subject to reduction by enzymes[10] or thiols such as glutathione (GSH) which is present in most cells at millimolar concentration (0.5–10 mM).[11] The reduction rate of the *cis* isomer of a *para*-substituted AMPB amino acid in a model tripeptide is about 100-fold higher than for the corresponding *trans* isomer.[11b]

To determine the stability of the AMPB switch unit in the peptide ligand **1** we incubated the *cis* form of the photoswitchable ligand at the photostationary state (pss) in buffer solution (pH 7.5) containing reduced glutathione (10 mM). After 1 h exposure to GSH no reduced material was detectable by LC–MS analysis (Figure 2, dashed line). Even after 16 h the reduced material amounted to only 5% (Figure 2, dotted line, signal marked with an arrow; Figure S3 in the Supporting Information). In addition, when changes in the UV/Vis spectra of the photoswitchable ligand were followed during irradiation in buffer solution (pH 7.5) containing GSH (10 mM), isosbestic points were retained, indicating the stability of the 3,4′-AMPB unit in the peptide ligand **1** (Figure S1 in the Supporting Information). As expected, the thermal *cis*→*trans* isomerization of the photoswitchable ligand **1** in the presence of 10 mM glutathione was

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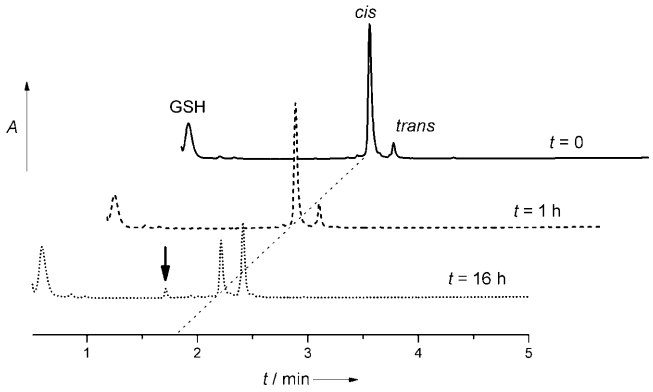

Figure 2. RP-HPLC profile (220 nm) of the photostationary state of the *cis* form (90% *cis* content) exposed to 10 mM GSH in buffer, pH 7.5 for different times: $t = 0$ (——), 1 (-----), and 16 h (•••••). The arrow indicates reduced peptide material (≈5%) after 16 h incubation.

found to be faster ($t_{1/2} = 3.1$ h) than without GSH ($t_{1/2} =$ 25 d),[1a] a fact that has been described previously also by others.[11b,12] However, for our studies the half-life is sufficient and the stability in the presence of GSH has been confirmed.

For imaging the cellular uptake of the photoswitchable ligand **1** we attached the fluorophore nitrobenzoxadiazole (NBD) to the lysine side chain (Figure 3a). Excitation of NBD is performed at $\lambda_{ex} = 488$ nm, a wavelength that should not interfere with the absorbance of the azobenzene-ω-amino acid. Either C2C12 myotubes or skeletal muscle fibers from *Flexor digitorum brevis* (FDB) were incubated for 15 min at 37 °C in buffer (7.5) with the NBD-labeled peptide ligand **2** (50 μM). The fluorescence scattered in both samples demonstrates that the peptide is capable of crossing the plasma membrane either of differentiated mouse muscle cells (Figure 3b) or small muscle organs displayed by the skeletal muscle fibers (Figure 3c).

Using an appropriate assay[13] for low-level NO detection based on the fluorescence of diaminofluorescein, we investigated the influence of in-cell photoswitching on nNOS-mediated NO release from C2C12 myotubes. Instead of 4,5-diaminofluorescein (DAF-2) we used the more pH- and photostable 4-amino-5-methylamino-2′,7′-difluorescein (DAF-FM)[14] to measure NO reliably in the supernatant of C2C12 myotubes. After incubation with the photoswitchable ligand **1** (20 μM) in its *trans* form and irradiation at $\lambda = 366$ nm (100 μW cm^{-2}), cells were stimulated with the calcium ionophore A23187 (1 μM) and incubated with DAF-FM (0.1 μM). Illuminated C2C12 myotubes in presence of the photoswitchable ligand **1** produced lower levels of NO than cells exposed to the *trans* form (Figure 4). This reduction of NO production by the *cis* form was found to be very similar to the reduction caused by the nNOS inhibitor 3-bromo-7-nitroindazole (20 μM) in a parallel experiment. Only the nNOS-derived NO burst was reduced, whilst the diminished NO production resulted from the formation by the two NOS isozymes eNOS and iNOS which are simultaneously expressed in C2C12 myotubes.[15] These NO studies suggest that the cell-permeable photoswitchable peptide ligand **1** controls the activity of sarcolemmal nNOS in mouse skeletal muscle cells by

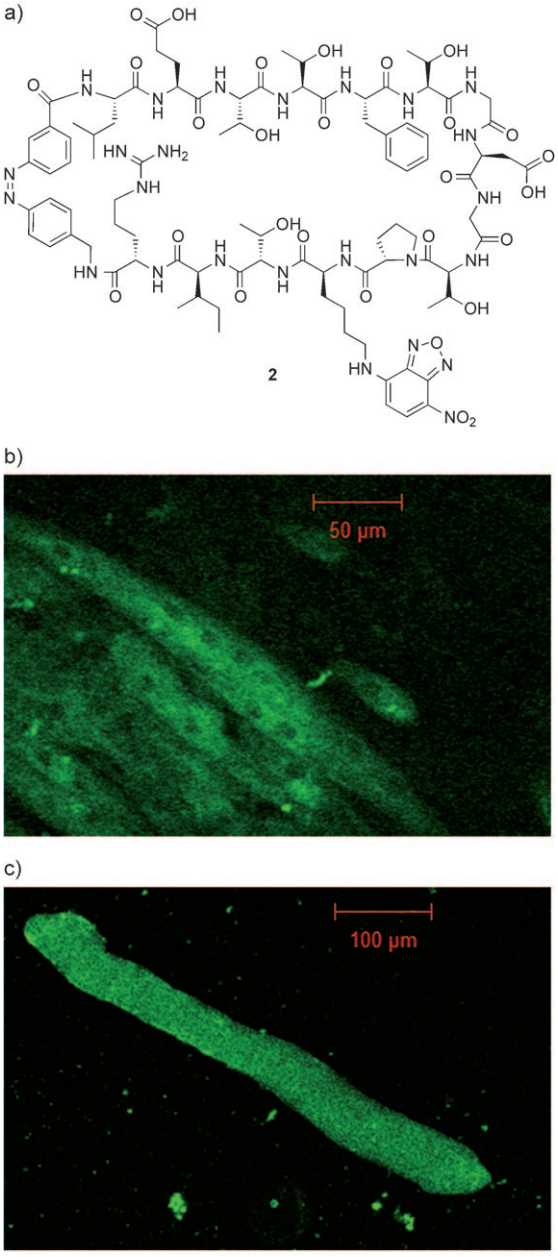

Figure 3. Uptake of a) NBD-labeled photoswitchable ligand **2** (50 μM) in b) C2C12 myotubes and c) in a single skeletal muscle fiber from *Flexor digitorum brevis* from three-month-old mice (wild-type). Excitation of NBD (nitrobenzoxadiazole) at $\lambda_{ex} = 488$ nm/λ_{em} = BP505–550 nm. BP = Bandpass filter.

selectively and specifically light-directed binding to α-1-syntrophin, disturbing the native interaction between syntrophin and nNOS.

To study the influence of light-induced inhibition of the syntrophin–nNOS interaction in a more complex biological system, we investigated the shortening of the sarcomere length of skeletal muscle fibers from *Flexor digitorum brevis* of three-month-old mice. *Flexor digitorum brevis* (FDB) known to produce NO in response to contraction[16] is the skeletal muscle in the middle of the sole of the feet. Without showing fatigue, fast-twitch FDB fibers were stimulated to

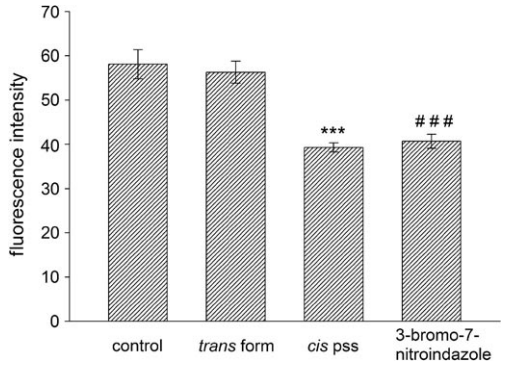

Figure 4. NO released from C2C12 myotubes detected by DAF-FM fluorescence ($\lambda_{ex}=495$ nm/$\lambda_{em}=515$ nm); cells exposed to $\lambda=366$ nm for 15 min or 3-bromo-7-nitroindazole released lower levels of nNOS-derived NO than cells in the presence of the *trans* form ($n=13$, 14 repetition). Significance analysis was performed using student's t-test. ***: $P<0.001$ (compared with *trans* form and control); ###: $P<0.001$ (compared with *trans* form and control).

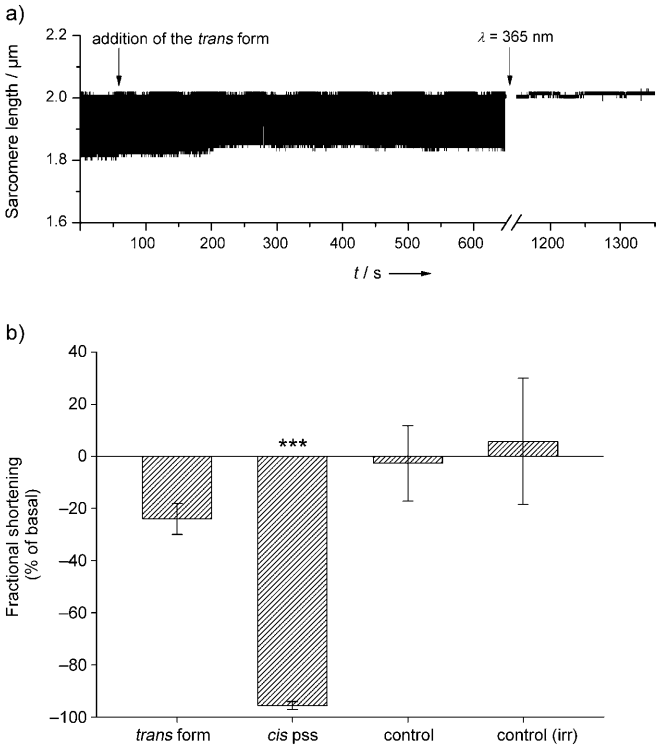

Figure 5. Functional effects of cell-permeable photoswitchable ligand **1** in living skeletal muscle fibers electrically paced (1 Hz). a) Original recordings of sarcomere shortening in the presence of the *trans* form and after illumination at $\lambda=365$ nm for 15 min. b) Statistical evaluation of the effect of photoswitching on fractional shortening (given in percent of basal contraction obtained before incubation with the peptide): the shortening amplitude of illuminated fibers containing the *cis* form of **1** is significantly reduced (96% to basal) while in the presence of the *trans* form the shortening amplitude was almost retained (24% to basal) ($n=6$). Control experiments include incubation with DMSO (0.1%) and illumination of the fibers at $\lambda=365$ nm ($n=4$). Significance analysis was performed using student's t-test. ***: $P<0.001$ (compared with *trans* form, control and control (irr)).

contract at 1 Hz. After a stable contraction rate had been reached, which is indicated by almost the same shortening amplitude of roughly 0.20 µm (Figure 5a), the *trans* form of the photoswitchable ligand **1** (50 µM) was added to the fibers. For the subsequent incubation no significant change in the shortening amplitude of the sarcomere length was observed (Figure 5a). The following illumination of the fibers at $\lambda=$ 365 nm resulted in a remarkable inhibition of the contractile function of the fibers displayed by the reduced shortening amplitude (96% to basal shortening amplitude) (Figure 5b). The same decrease in shortening amplitude was obtained when fibers were treated with the nNOS inhibitor 3-bromo-7-nitroindazole (data not shown) indicating the interference within the nNOS pathway and activity. The muscle fibers showed neither hypercontraction nor sarcomere damage after the whole procedure. Control experiments with fibers in the absence of the peptide demonstrated that illumination at $\lambda=$ 365 nm has no effect on contractile function in contrast to that observed for aortic smooth muscles which showed photorelaxation when irradiated in the near-UV region (≈310 nm).[17] The data obtained here suggest that as a result of the perturbation of the syntrophin–nNOS interaction nNOS may be lost from the sarcolemma to cause significantly reduced muscle performance. Irradiation of the muscle fibers in the presence of the *cis* form at $\lambda=435$ nm to induce photoisomerization to the *trans* form of **1** showed no re-establishment of muscle fiber contraction. As the irradiation of **1** at approximately 435 nm regenerates only about 75% of the *trans* form, the resulting concentration of about 12.5 µM for the remaining *cis* form could be still sufficient to avoid formation of the syntrophin–nNOS complex. Therefore, we studied the system in vivo at the reduced initial concentration of **1** (12.5 µM). Although the *cis* form showed under these conditions a significant but incomplete inhibition of muscle contraction (data not shown), illumination at $\lambda=435$ nm showed again no re-establishment of fiber contractility. Assuming reversibility of the binding of **1** to syntrophin as shown in vitro (see Figure S4 in the Supporting Information), the nonreversibility of the process in vivo may result from nonreversible events in the fibers caused by disruption of the syntrophin–nNOS complex.

The results demonstrate that our recently described nNOS-derived, photoswitchable peptide ligand of α-1-syntrophin, which was shown to bind to the PDZ domain of α-1-syntrophin in the *cis* but not in the *trans* form of the incorporated azobenzene switch, can be used also in living systems such as skeletal muscle fibers. Under physiological conditions, where the peptide ligand is sufficiently stable, photoisomerization to the *cis* form resulted in an almost complete inhibition of the electrically stimulated fiber contraction whilst the *trans* form had almost no effect on the muscle fiber shortening. Light-directed binding of the *cis* form of the photoswitchable ligand to syntrophin may be expected to inhibit the syntrophin–nNOS interaction in the skeletal muscle followed by the dislocation of nNOS from the sarcolemma. This may result in reduced NO release from skeletal muscle cells and in light-controlled muscle contraction, underlining the impact of nNOS localization by α-1-syntrophin for muscle activity. Strategies to inhibit nNOS

activity and localization under high spatial and temporal control may be beneficial for investigating muscle diseases as well as for maladies like stroke. Very recently the disruption of the familiar PDZ/PDZ interaction of nNOS to PSD-95 was realized by melting the β-finger structure in nNOS to treat cerebral stroke.[18] Therefore nNOS is becoming increasingly interesting as a therapeutic target.

The ability to activate and release drugs by light may make it possible to investigate and treat diseases by remote control; however, the range of the used tissue has to be considered.

Experimental Section

The photoswitchable peptide ligand **1** was prepared using standard Fmoc-based solid-phase synthesis as previously described (see the Supporting Information for Ref. [1a]). NBD labeling[19] was realized by adding 0.10 mg 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (50 μmol) in MeOH (4.0 mL) to a solution of 1.0 mg photoswitchable peptide **1** (50 μmol) dissolved in $NaHCO_3$ at pH 8.5 (1.0 mL). The reaction mixture was warmed up to 55 °C and stirred at RT for 1 h. Most of the solvent was evaporated and after acidification to pH 1.5 with trifluoroacetic acid (1.0 mL), the peptide was precipitated by addition of cold Et_2O. Composition of the NBD-labeled peptide **2** was confirmed by ESIMS (ES+) $[M+2H]^{2+}$ = calcd: 1009.9650 (mono-isotopic); obsd:1009.9237.

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