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Cytoplasmic nucleic acid-based XNAs directly enhance live cardiac cell function by a Ca^{2+} cycling-independent mechanism via the sarcomere

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Abstract

Nucleic acid - protein interactions are critical for regulating gene activation in the nucleus. In the cytoplasm, however, potential nucleic acid-protein functional interactions are less clear. The emergence of a large and expanding number of non-coding RNAs and DNA fragments raises the possibility that the cytoplasmic nucleic acids may interact with cytoplasmic cellular components to directly alter key biological processes within the cell. We now show that both natural and synthetic nucleic acids, collectively XNAs, when introduced to the cytoplasm of live cell cardiac myocytes, markedly enhance contractile function via a mechanism that is independent of new translation, activation of the TLR-9 pathway or by altered intracellular Ca^{2+} cycling. Findings show a steep XNA oligo length-dependence, but not sequence dependence or nucleic acid moiety dependence, for cytoplasmic XNAs to hasten myocyte relaxation. XNAs localized to the sarcomere in a striated pattern and bound the cardiac troponin regulatory complex with high affinity in an electrostatic-dependent manner. Mechanistically, XNAs phenocopy PKA-based modified troponin to cause faster relaxation. Collectively, these data support a new role for cytoplasmic nucleic acids in directly modulating live cell cardiac performance and raise the possibility that cytoplasmic nucleic acid - protein interactions may alter functionally relevant pathways in other cell types.

Keywords

DNA; RNA; contraction; sarcomere; troponin

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Introduction

Recognition of the functional diversity of nucleic acids in biology has increased dramatically over the past decade. The annotation of cytoplasmic short non-coding miRNAs and long non-coding RNAs is vast and continues to expand^{1, 2}. Along with diverse RNAs, endogenous nuclear and mitochondrial DNA byproducts can also accumulate in the cytoplasm of cells^{3, 4}. The possible functional consequences of these cytoplasmic localized nucleic acids, if any, has not been established.

In addition to investigating whether cytoplasmic nucleic acids may directly modify cellular biological processes, exogenous nucleic acid derivatives have been developed with the aim to alter cell function. These include siRNAs, shRNAs, and exon skipping oligonucleotides designed to target specific RNA/DNA sequences to directly alter gene expression^{5, 6}. Interactions between cytoplasmic nucleic acids and cellular proteins are well known to affect key aspects of gene transcription and translation⁷. These examples highlight the emerging complexity of cytoplasmic nucleic acids in biology with great interest now focused on deciphering their cellular function⁸. Given the emergence of the complexity of cytoplasmic non-coding nucleic acids, an intriguing possibility follows that endogenous non-coding RNAs/DNAs may directly interact with cytoplasmic proteins or protein networks to directly modulate live cell functional performance.

We now show evidence of non-coding natural and synthetic nucleic acids, termed XNAs, functioning in the cytoplasm to cause direct effects on live cell performance. Using intact adult cardiac muscle cells studied under physiological conditions as an experimental template, our results indicate that oligo-based XNAs, independent of new translation, can directly modulate physiologically relevant biological processes in the cytosol. Specifically, findings show that XNAs bind with high affinity to the key sarcomeric regulatory complex troponin and myocyte relaxation function is enhanced via a mechanism independent of new translation, activation of the TLR-9 pathway or by alterations in intracellular Ca²⁺ cycling. These results support a mechanism whereby cardiac troponin structure-function is altered by XNAs phenocopying the PKA-dependent troponin modification known to elicit faster relaxation⁹. Because cardiac relaxation performance is a tightly controlled process, cytoplasmic XNAs could represent a new pathway to manipulate cardiac contractile performance.

Methods

Ventricular myocyte isolation and primary culture:

Adult rat ventricular myocyte isolation was performed as previously described¹⁰. Briefly, adult female rats were anaesthetized by inhalation of isoflurane followed by i.p. injection of heparin (15,000 U/kg) and Fatal Plus (150 mg/kg). Following enzymatic digestion by retrograde perfusion with collagenase and gentle trituration of the cardiac ventricles, cardiac myocytes were plated on laminin-coated glass coverslips (2×10⁴myocytes/coverslip) and cultured in M199 media (Sigma, supplemented with 10 mmol/L glutathione, 26.2 mmol/L sodium bicarbonate, 0.02% bovine serum albumin, and 50 U/ml penicillin-streptomycin, with pH adjusted to 7.4, additionally insulin (5µg/ml), transferrin (5µg/ml) and selenite

(5ng/ml) (ITS) were added (Sigma I1884). One hour after plating, nonadherent cells were removed and fresh M199 was applied.

ssDNA transfection:

Day one isolated adult cardiomyocytes were transfected with ssDNA via Lipofectamine 2000 transfection reagent according to the manufacturer's protocol (Invitrogen). ssDNA of varying length was mixed with 5 µl Lipofectamine 2000 and applied to the cells in 1 ml Optimem. The final concentration of ssDNA was 100 nM in all studies. The transfection was allowed to proceed for 2 hours and then fresh media was applied until functional measures were conducted. No DNA controls with Lipofectamine and no Lipofectamine controls with ssDNA were used to control for Lipofectamine effects.

ssDNA used:

80mer: 5'TTGGGGAGGGGCACTGGGCAGTGTAATTTACGAAAGCGAGTTGGGGAGGGGCACTGGGCAGTGTAATTTACGAAAGCGAG-3'

40-mer: GTA GAG ATT CGT ATT TTG GGG AGA AGC GGC CGG AAG CGG A

Control DNA: 5 nucleotides: GCT TG

10, 20, 35, 50, 80-mer for length dependence: completely random sequence ssDNA synthesized to be a combinatorial library

50-mer: Random sequence single stranded DNA

L-DNA: 50-mer random sequence single stranded enantiomeric DNA

RNA: 50-mer random sequence single stranded RNA

PTDNA: 50-mer random sequence phosphorothioate backbone single stranded DNA

OMRNA: 50-mer random sequence of 2'-O-methyl RNA

Poly-T DNA: 50 thymine bases

dsDNA: 40-mer 5' CTCGCTTTTCGTAAATTACACTGCCAGTGCCCCCTCCCCAA 3'

Contractility measurements in single intact myocytes:

Sarcomere length dynamics were performed as previously described¹⁰. Briefly, cover slips containing single isolated myocytes, day one after isolation, were placed on an inverted microscope (Nikon, Eclipse TE2000) and electrically stimulated at 1 Hz in a 37°C media bath. Tyrode's Solution pH 7.4 (Calcium 1.8 mM) was used for the media bath. Sarcomere length recordings were collected (1000Hz) using a CCD camera (MyoCam, IonOptix). Myocytes that did not follow the pacing protocol (1 Hz) were excluded. Sarcomere length shortening and relaxation kinetics were calculated using IonOptix software. For calcium measurements 2 µM Fura-2-AM was loaded into cells for 10 minutes at room temperature.

The cells were then washed and visualized 15 minutes later to allow for de-esterification. Measurement of the 360/380 ratio was calculated using the Ionoptix software. For isoproterenol stimulation, 10 nM isoproterenol in Tyrodes pH 7.4 was added to the cells and 5 minutes later calcium and sarcomere dynamics were measured simultaneously. For cyclohexamide (CHX) experiments, cells were pretreated with 100 μ M CHX for ten minutes, then cells were treated with XNA as above with CHX present. For all experiments two to five independent myocyte preps were used, with N=15–60 myocytes analyzed in total.

Western blotting:

Adult cardiac myocytes +/- ssDNA and +/- 10 nM isoproterenol as above were harvested in RIPA buffer containing protease and phosphatase inhibitors. Samples were handled and run on SDS-PAGE and transferred to PVDF membrane as previously described. After blocking in Li-Cor blocking buffer, membranes were probed with total cTnI antibody (4C2; 1:4000, Advanced ImmunoChemical, Inc) and S23/24 phospho-cTnI (4004; 1:1000, Cell Signaling Technology). This antibody has been highly used in the field by many groups to track PKA-dependent phosphorylation status of cTnI in cardiac muscle^{11–15}. Indirect immunodetection was carried out using a fluorescently labeled anti-mouse secondary antibody (Li-Cor, IRDye 680 conjugated affinity purified; 1:10,000). Western blot analysis was accomplished using the infrared imaging system, Odyssey (Li-Cor, Inc.) and images analyzed using Odyssey software v3.0.

CHX control:

Adult cardiac myocytes were transduced with a CMV-GFP Adenovirus vector at 100 MOI one day after isolation. Myocytes were monitored for GFP+ cells via fluorescence microscopy. At six hours post transduction the first few GFP+ cells were visualized indicating the beginnings of new protein (GFP) translation, and at this time point CHX at 100 μ M was added to the CHX group. Three hours after CHX administration cells were imaged and analyzed for GFP+ rod shaped cells. Nontransduced cells served as the negative control for GFP+ cells. Three independent wells with multiple images in each well were used. Total rod shaped cells were counted and GFP+ rod shaped cells were counted. Percent of rod shaped cells per image were averaged. N=54 rod shaped cells per group.

Fluorescence of 40-mer-AF:

40-mer ssDNA labeled with Alexafluor 594 was transfected at 100 nM for two hours into adult ventricular cardiac myocytes. Cells were then treated with blebbistatin (10 μ M) to preserve morphology and permeabilized in 0.1% Triton-X 100 PBS for 5 minutes. Cells were washed three times and fixed with 4% paraformaldehyde for 15 minutes. Coverslips were mounted onto glass slides and imaged on a Zeiss Axioskop LSM 510 laser scanning confocal microscope. No DNA served as a control. Images shown are at 40X with a blowup in the final panel.

XNA-Sarcomere binding assay:

Adult rat cardiac myocytes were isolated and plated as above. One day after isolation coverslips were fixed in 4% paraformaldehyde for 10 minutes and then permeabilized in 0.1% Triton-X 100 PBS for 5 minutes. XNA-Alexafluor 594 labelled 40-mer was incubated at 50 μ M in 25 mM KCl for one hour. The coverslips were washed two times and then fixed again as above. The coverslips were blocked with PBS 5% BSA for one hour and α -actinin primary antibody (EA53) 1:500 in PBS 5% BSA was incubated for one hour. Cells were washed three times in PBS 0.1% Triton-X 100 and Alexafluor 488 goat anti mouse secondary was incubated at 1:1000 for one hour. Cells were washed as before and then mounted with Prolong Diamond antifade mountant. Images were acquired on a Nikon A1R with 63x water immersion objective.

Protein Purification:

Recombinant human troponin proteins were purified from B121 (DE3) pLysS Rosetta E. coli (Millipore #70956) transformed by pET200D plasmids (ThermoFisher #K20001) containing each individual human troponin subunit. E. coli were grown overnight in auto-induction media; the resultant bacterial pellet was lysed by sonication and purified by immobilized cobalt ion affinity chromatography. The 6xHis tag on each subunit was removed by enzymatic digestion with tobacco etch virus protease and then further purified by immobilized cobalt ion affinity chromatography to yield tag-free human troponin subunits. The three troponin subunits were then either dialyzed into 150mM KCl, 50mM Tris, 2mM MgCl₂, pH 7.2 at 4°C or combined in equal molar amounts and denatured in 6M urea. Reconstitution of the troponin complex was done by dialyzing the denatured proteins against 150mM KCl, 50mM Tris, 2mM MgCl₂, pH 7.2 at 4°C. Troponin complex was confirmed by native-PAGE and stored at -80°C.

Purification of bovine troponin complex followed the method of Bier et al¹⁶. Briefly, frozen bovine heart tissue was pulverized and then mixed with 400mM LiCl and pH was adjusted to 5.1. The tissue was then filtered through cheesecloth and pH was adjusted to pH 7.5 by Tris base. 19%(w/v) ammonium sulfate and .084% (w/v) sodium bicarbonate were added, the slurry was then centrifuged at 8000rpm for 20min and the supernatant was reserved. Additional 8.5% (w/v) ammonium sulfate was added to the supernatant and then centrifuged at 8000rpm for 20min and the pellet was reserved. The pellet was then resuspended in 50mM TrisHCl, 1mM DTT, 1mM EGTA pH 8.0 and dialyzed repeated against the same solution overnight. The solution was then centrifuged at 40,000rpm for 30min and the supernatant was reserved. The supernatant was applied to a DEAE cellulose column and eluted against a 300mM NaCl gradient to yield the intact troponin complex. Troponin complex was identified by SDS-PAGE; then was pooled at stored at -80°C.

For purification of actin, 1g of rabbit skeletal acetone powder was mixed and allowed to swell with 20ml of buffer containing 5mM Tris pH 7.5, 200mM MgCl₂, 0.5mM ATP for 30min on ice. 30mM KCl was added and the solution incubated at room temperature for 2 hours. The solution was then centrifuged at 230,000g for 1 hour. The resultant pellet was rinsed in the above buffer and homogenized and used for experiments within 10 days.

Rabbit skeletal myosin was purified as described by Margossian et al., and stored at -20°C in 10 mM Tris, 600 mM KCl with 50 % glycerol¹⁷. Prior to digestion with chymotrypsin to yield myosin S1, the frozen myosin was dialyzed against 20 mM Tris pH 7.0, 30 mM KCl, centrifuged to sediment the resulting myosin filaments. The resultant pellet was then reconstituted in 20 mM Tris pH 7.0, 0.6M KCl, 2 mM MgCl_2 . Myosin was then digested with α -chymotrypsin (SigmaAldrich #C4129) for 10 minutes at room temperature and then dialyzed into 10 mM Tris pH 7.0 with 150 mM Sucrose prior to storage at -80°C .

Affinity Capillary Electrophoresis (ACE):

A Beckman Coulter (Fullerton, CA) commercial CE system (P/ACE MDQ) was used for all capillary electrophoresis experiments. Oligonucleotide sequences were labeled with fluorescein (FAM) and signals were detected using laser induced fluorescence with $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 520 \text{ nm}$. The fluorescently labeled oligonucleotides were kept at a constant concentration of 20 nM and mixed with increasing concentrations of the target protein for analysis. Samples were injected using hydrodynamic injection of 1 psi for 4 secs into a 40 cm $\times$ 50 μm fused silica capillary (Polymicro Technologies, Phoenix, AZ) for separation. ACE separations were performed at 30kV for 12 minutes. Electropherograms were analyzed using 32 Karat and Cutter 7.0¹⁸. Peak heights and/or area of the free oligonucleotide peak were used to determine the K_d , according to¹⁹:

$$f_b = \frac{c}{1 + K_d} \cdot \frac{1}{[P]_t - 0.5([D]_t + [P]_t + K_d - (([D]_t + [P]_t + K_d)^2 - 4[D]_t[P]_t)^{1/2})} \quad (2)$$

where f_b is the bound fraction; c is the maximum bound fraction; $[P]_t$ and $[D]_t$ are total concentrations of protein and oligonucleotides. The denominator represents the free concentration of protein. ssDNA was a 50-mer random sequence, dsDNA was a 40-mer sequence 5' CTCGCTTTCGTAAATTAACTGCCCCAGTGCCCCCTCCCCAA 3', troponin subunits were tested with 80-mer random sequence.

Fluorescence Polarization (FP):

A Synergy™ 2 Microplate Reader from BioTek Instruments, Inc. (Winooski, VT) was used to perform FP experiments. Fluorescein (FAM) fluorescently labeled oligonucleotides were used for experiments and sample conditions were identical to ACE sample conditions. Filter settings for the FP experiments were as follows: $\lambda_{\text{ex}} = 485 \pm 20 \text{ nm}$, $\lambda_{\text{em}} = 528 \pm 20 \text{ nm}$. 15 μL aliquots of each sample was loaded into a black Corning 384 well plate (3540 microplate from Corning Incorporated, Corning, NY) or 150 μL of sample was loaded into a black Corning 96 well plate for polarization experiments. Intensities of the parallel and perpendicular light were measured using a sensitivity setting of 120. The polarization values were calculated from the parallel and perpendicular intensity values. The g factor was used for calibrations. This calibration was calculated using the Gen 5™ software for the instrument (BioTek Instruments, Inc., Winooski, VT). The bound fraction of oligonucleotide was determined according to the following and for K_d determination:

$$f_b = \frac{P - P_o}{P_m - P_o} \quad (3)$$

Where f_b is the bound fraction; P , P_m and P_o are the measured polarizations of a sample, complex, and free oligonucleotide, respectively. The plateau value used is the polarization of the complex (oligonucleotide and target protein) (P_m) and is at saturating protein concentrations. K_d was determined by fitting data to equation (2) using Origin 9.1.

Statistics:

All results are expressed as mean $\pm$ SEM. Multi-group comparisons were assessed using one way analysis of variance (ANOVA) with Bonferroni post-hoc test with $P < 0.05$ considered statistically different.

Results

Cytoplasmic nucleic acid XNAs directly alter live cardiac myocyte function

To test for the potential direct effects of XNAs on live cell function, we first used single stranded DNA (ssDNA) transfected into freshly isolated adult ventricular myocytes. Delivery of Alexa-fluor labeled ssDNAs demonstrated the localization of XNAs in the cytoplasm of the cardiac myocytes (Fig. 1B). Here, the cytosolic staining is striated in appearance consistent with a sarcomeric labeling pattern. Delivery of random ssDNAs, with lengths of 40 to 80 nucleotides, produced a highly consistent maximal acceleration in the early phase of cardiac myocyte relaxation, with significantly faster times to return to baseline sarcomere length, while showing no change in peak amplitude of contraction (Figure 1D–E; Fig. S1–S6). Shorter ssDNAs, < 35 mer, had no effect, indicating a steep length dependence of ssDNAs on intact cardiac myocyte function (Fig. 1E). The effect to speed relaxation was evident 2 hours after transfection and was largely gone by 24 hours (Figure S4–S5). Analysis of the concentration and length-dependence of this effect showed that 100 nM in the transfection mixture and at least 40 nucleotides are necessary to cause accelerated relaxation (Fig 1E; Fig. S1–S3). Due to the inherent variation in myocyte transfection efficiency with different XNA concentrations, experiments designed to test the concentration-dependence of XNAs showed some variability; nonetheless, in all cases, it was evident that 100 nM was the lowest effective concentration to elicit a highly reproducible maximal effect to significantly accelerate myocyte relaxation (Figure S1–S2). The XNA length-dependent effect was identical using either ordered or random sequence oligonucleotides, indicating a non-sequence dependent effect (Fig. 1D).

To determine whether ssDNAs have effects on myocyte intracellular Ca^{2+} handling we monitored twitch Ca^{2+} transient dynamics with the fluorescent indicator Fura-2 (Figure 1F–H). In comparison to control ssDNAs (5-mer) and no DNA controls, ssDNA had no effect on the intracellular Ca^{2+} transient amplitude or kinetics (Fig. 1G–H). Taken together with the sarcomere length contraction data, this is evidence that ssDNAs speed relaxation in a Ca^{2+} transient-independent manner. Previous work has shown that random sequence ssDNA can

bind with high affinity to phospholamban resulting in increased SERCA activity in solution studies^{8, 20}. However, the Ca^{2+} -independence of the relaxation effect observed here in intact cardiac myocytes demonstrates that phospholamban-mediated SERCA inhibition and overall Ca^{2+} handling are not affected by ssDNAs in the setting of intact adult cardiac myocytes activated under physiologically relevant intact excitation-contraction coupling conditions.

We next tested ssDNA's effects on contraction in the presence and absence of β -adrenergic stimulation. Surprisingly, Isoproterenol (Iso) had no additional effect to further accelerate the speed of myocyte relaxation caused by ssDNAs (Fig. 1I and K; Fig. S7). In addition, the magnitude of Iso-mediated increased contraction amplitude was not affected by ssDNAs (Fig. 1J). This is evidence that ssDNAs elicited a maximal physiological effect in terms of accelerating cardiac relaxation performance. In cardiac muscle, it is well known that Iso-mediated faster myocyte relaxation is caused in part by hastening Ca^{2+} uptake kinetics, which were unchanged by ssDNA (Fig 1L–N; Fig. S8). Phosphorylation of cardiac troponin I (cTnI) is another key regulator of β -adrenergic stimulated enhanced relaxation⁹. To determine if phosphorylation of cTnI is altered by ssDNAs we probed for phosphorylation of S23/24 of cTnI via Western blotting. Results showed no effects of ssDNAs on the levels of phospho-cTnI, +/- Iso (Fig. S9) providing evidence that the phosphorylation status of S23/24 of cTnI is unaltered by ssDNA. This does not exclude other phosphorylation sites from being affected by ssDNA. It is also worth noting the physiologic functional effect of isoproterenol was not altered by ssDNA: the peak SL amplitude and relaxation rates with Iso were the same +/- ssDNA. Stated differently, ssDNA did not further accelerate relaxation rates in the presence of Iso, suggesting a physiological limit on relaxation performance.

XNA structure and mechanism of action studies

Next, we tested whether modifications in nucleic acid structure/composition would have cellular physiological effects similar to ssDNAs. Accordingly, we tested double stranded DNAs (dsDNA), the enantiomer of ssDNA (L-DNA), phosphorothioate backbone-modified DNAs (PS), and RNAs via 2'-o-methyl RNAs (2-OMe) as a stable RNA moiety. Here, dsDNA, L-DNA, PS and 2-OMe RNAs all had the same effect to maximally accelerate myocyte relaxation similar to ssDNAs (Fig. 2A–D). Taken together, these XNAs, including single or double stranded DNAs and chemically modified DNAs and RNAs, provide evidence of significant flexibility in the nucleic acid ribose sugar structure to elicit the cellular physiologic effect of enhanced relaxation.

The transfected XNAs were detected in the nucleus (as expected for a transfection reagent) and in the cytoplasm of the myocytes (Fig. 1B); therefore, we investigated whether the effects observed required new protein production. Thus, to further probe the mechanism, we used cycloheximide to inhibit cellular translation in the XNA-treated myocytes. In a control experiment, we validated, under our conditions in myocytes, that cycloheximide inhibited new protein translation. This was demonstrated by analyzing new GFP expression after acute gene transfer (Fig S10). Results following XNA delivery showed that treatment of adult cardiac myocytes with cycloheximide had no effect on the XNA-mediated increased speed of relaxation (Fig. 3B). This is evidence that XNA effects are direct and do not require translation and new protein expression.

We next tested whether XNAs provoke cellular innate immunity to cause the observed contractile effects. Toll-like receptor 9 (TLR-9) is the primary ssDNA receptor in the innate immunity system^{21, 22}. In cardiac myocytes, TLR-9 activation results in metabolic changes while in immune cells, activation elicits cytokine production²². TLR-9 recognizes unmethylated CpG pairs to differentiate between foreign DNA and self-DNA²¹. To test whether the observed cellular effects here were through TLR-9 activation we used ssDNAs consisting of all thymines (Poly T). Poly T ssDNAs resulted in the same enhancement of relaxation as did control random ssDNAs, and this is evidence that TLR-9 signaling is not required to hasten myocyte relaxation (Fig. 3C). In addition, the XNA length-dependence data (Fig. 1E) further supports this interpretation, as short oligonucleotides (<35 mer) activate the TLR-9 pathway, but these shorter XNA's have no effect on myocyte performance (Fig. 1D–E).

XNAs bind with high affinity to the sarcomere regulatory troponin complex

The independence of faster relaxation by XNA's from new protein translation and innate immune receptor activation is evidence of a direct action of XNA's on cytoplasmic proteins/pathways in the cell. Physiologically, there are two well established processes that could account for XNA-mediated acceleration in live cell relaxation rates: 1) through increased rates in Ca^{2+} sequestration via SERCA-PLN and/or 2) by direct modification in sarcomere performance^{9, 23}. It is well established that relieving the PLN-mediated inhibition of SERCA2a function in live cardiac muscle (e.g., PLN inactivation) results in markedly faster Ca^{2+} transient decay rates, along with increased magnitude of contraction owing to increased SR Ca^{2+} loading²⁴. However, the observations here of Ca^{2+} transient-independence of the enhanced relaxation rates with no change in contractile amplitude (Fig. 1) provide evidence against XNAs altering the Ca^{2+} cycling pathway in intact adult cardiac myocytes.

This finding, together with the XNA sarcomeric localization denoted by the striated labeling pattern (Fig. 1B), provides evidence that ssDNAs have a direct effect on the myofilament-based contractile apparatus. To further test this possibility, we determined whether XNAs could bind with high affinity to purified sarcomeric proteins, as suggested previously²⁵. Fluorescence polarization and capillary electrophoresis data showed low nM affinity of random sequence ssDNA to both the bovine and human sarcomeric troponin complex (Fig. 4A and C). In comparison to troponin, purified myosin S1 showed much weaker affinity to ssDNA (Figure 4; Fig. S11; Table S1). Because dsDNA has a similar physiologic effect as ssDNA we next tested sarcomere components binding to dsDNAs. Here dsDNAs bound to the troponin complex with the same high affinity as ssDNAs, however, myosin binding was markedly weaker (Fig. 4B). In addition, for SERCA2a/phospholamban it has been shown that dsDNAs have no effect on in vitro SERCA activity, indicating no functional binding effect of dsDNA with phospholamban²⁰.

In further dissecting the main components of the cardiac sarcomere, data show that purified actin has no functional binding affinity for XNAs (Table S1). Interestingly, as above, the heterotrimeric troponin (Tn) complex, the well known essential regulatory switch of contraction, showed high binding affinity and further analysis of the individual Tn subunits

showed the highest affinity for cTnI, moderate affinity for cTnT and no binding to cTnC (Fig. 4C). Because none of the individual Tn subunits had the same high affinity demonstrated by the whole Tn complex, this indicates that the highly ordered structural features of the Tn complex²⁶ are required for high affinity XNA binding. To gain further insight into the mechanism of binding, salt concentrations were varied up to 150 mM KCl. The Tn complex showed a steep salt dependence to XNA interaction indicating electrostatics as a key component underlying high affinity Tn-XNA interaction (Fig. S11). In this context, to further test whether XNAs can bind troponin in the context of the sarcomere, we incubated permeabilized cardiac myocytes with XNA-Alexafluor-594 40-mer along with α -actinin to label Z-lines. Results show that XNA AF-40 mer labels the entire length and width of the myocyte in a striated pattern localized between the Z-lines, providing evidence of XNA binding to the sarcomere (Fig 4). Taken together, these data suggests that XNA can bind to the Tn complex with high affinity in the context of the sarcomere.

Discussion

We report here that cytoplasmic nucleic acids, termed XNAs, can localize to the sarcomere, bind with high affinity to the troponin regulatory complex and directly modulate intact adult cardiac myocyte relaxation performance in a Ca^{2+} cycling independent manner. Because XNAs bind with high affinity to Tn, and Tn is a well known key component regulating myocyte function, including fast relaxation⁹, we posit that XNA-Tn interaction provides the mechanism, at least in part, for XNA-mediated fast relaxation. The high affinity for both ssDNA and dsDNA for troponin highly suggests that troponin is the primary target in the sarcomere to facilitate faster relaxation as both XNA's elicit similar cellular effects. Our findings, however, do not exclude other potential sarcomeric targets that may also contribute to XNA-mediated accelerated myocyte relaxation, along with troponin.

Mechanistically, the steep length-dependence of XNAs (minimum 40 mer) suggests XNA binding the length of the Tn complex is critical, which is on the order of 25 nm²⁶, and this is the same length scale calculated for 40 mer XNAs²⁷. Troponin concentration in cardiac muscle has been estimated at ~28 μM ²⁸. The concentration of XNA in the cytoplasm in the present study is not known; however, it is likely much higher than the 100 nM concentration in the transfection mixture given the detection of labeled XNAs in the cytoplasm. If XNA concentration in the cytoplasm is lower than the troponin content, the highly cooperative cardiac myofilament regulatory system could readily account for the physiological findings reported here based on the high cooperativity between functional units along the thin filament²⁹. Results further show that XNA-troponin binding is salt dependent, indicating an electrostatic interaction, and that binding affinity is highest for the whole troponin complex. Because structural domains are more ordered in the troponin complex than in the individual subunits this suggests XNA binding to a specific structured state in the complex²⁶. Elucidating the precise binding site(s) will require future detailed investigation.

In addition, it has been shown that PKA-based post-translation modification of Tn is sufficient to produce Ca^{2+} -independent faster relaxation in cardiac myocytes⁹. We propose that XNAs, shown here not to affect the PKA-dependent phosphorylation content in troponin per se, bind Tn to induce faster relaxation in a manner mechanistically similar to that caused

by PKA-mediated phosphorylation of Tn. Phosphorylation of cTnI leads to increased relaxation through a structural transition wherein the troponin I N-terminal domain detaches from cTnC. Our findings are therefore consistent with XNAs phenocopying PKA-induced troponin alterations that in turn speed myocyte relaxation.

There is growing interest in deciphering the potential direct effects of non-coding nucleic acids on cytosolic biological processes within the cell³⁰. Cytoplasmic nucleic acids are thought to affect cell function by altering gene expression via disruption in translational output^{1, 31}. There is increased speculation that the functional repertoire of cytoplasmic nucleic acids may extend beyond gene regulation to include potential direct effects on diverse processes within the cell³⁰. Our findings support an expanded role for cytosolic DNA/RNAs to include their direct interaction with key protein machinery essential for normal cell performance. Cardiac myocyte relaxation is a highly regulated and essential process of the myocyte contractile cycle²³. Given the central role of troponin in regulating the sarcomere and modulating relaxation performance, our findings support a model wherein length- (but not sequence) dependent cytoplasmic XNAs directly bind to troponin to directly cause enhanced cardiac myocyte relaxation performance.

Cardiac relaxation function is an essential and tightly controlled parameter of normal heart performance. In a myriad of both acquired and inherited cardiac disorders, myocyte relaxation function can be impaired leading to compromised heart pump performance. The findings here that XNAs can markedly accelerate relaxation, and to an extent indistinguishable from that achieved by β -adrenergic stimulation, presents a potential unique pathway to directly enhance myocyte function independent of altering intracellular Ca^{2+} cycling. Further, there is evidence that cytoplasmic XNA content can vary in cardiac disease states in the form of mitochondrial DNA³², non-coding RNA³³ or circular RNA³⁴. In addition, cell-free XNA and exosomal XNA are altered in disease, with the potential to alter cytosolic XNA through cell-cell communication^{35–37}. These findings, together with the new results reported here, raise many new questions for future studies centered on assessing myocyte to whole heart performance in both acquired and inherited cardiac disease models.

Taken together, data here support a new role for nucleic acids in directly modifying the dynamic function of the multimeric sarcomeric contractile machinery in live cardiac myocytes. Conceptually, we speculate that other cellular processes and cell types may also be affected by nucleic acids to impact function of diverse biologically relevant individual proteins or protein networks within the cell. In addition, these findings have potential implications for studies involving the delivery of exogenous oligonucleotides to cells that, in addition to modifying desired targeted gene expression/translation processes, could have direct physiologically relevant effects in modulating protein function including, as shown here, cardiac myocyte performance. Collectively, these findings support a broadening of the functional role for cytoplasmic RNA/DNA/XNA milieu and further highlight the emerging complexity of nucleic acids in directly affecting live cell function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. ssDNA enhances relaxation of adult cardiac myocytes. A. Structural representation of deoxy-ribonucleotide from 5–80 bases. B. Fluorescence of Alexafluor conjugated 40-mer ssDNA transfected into adult cardiac myocytes. No DNA control (left panel) and blowup (right panel). C. Representative traces of sarcomere dynamics $\pm$ 80-mer ssDNA normalized to baseline sarcomere length and peak amplitude. D. Time to return to 25% baseline sarcomere length from peak amplitude of contraction as a measure of relaxation for no DNA, 5-mer ssDNA and 80-mer ssDNA. E. Length dependence of the relaxation measure time to return to baseline 25% for random sequence ssDNA. F. Representative traces of Fura-2 measured calcium transients in adult cardiac myocytes $\pm$ 80-mer ssDNA. G. Calcium transient peak amplitude as a 360/380 ratio for No DNA control, 5-mer ssDNA and 80-mer ssDNA. H. Calcium transient time to decay 25% for no DNA control, 5-mer ssDNA and 80-mer ssDNA. I. Representative traces for sarcomere dynamics $\pm$ isoproterenol (iso) and $\pm$ 80-mer ssDNA normalized to baseline and peak amplitude. J. Peak amplitude of contraction $\pm$ isoproterenol (iso) and 80-mer ssDNA. K. Relaxation measure time to return to 25% baseline sarcomere length $\pm$ isoproterenol (iso) and 80-mer ssDNA. L. Representative traces of calcium transients $\pm$ isoproterenol (iso) and $\pm$ 80-mer ssDNA normalized to baseline and peak amplitude. M. Calcium transient amplitude for isoproterenol treated or untreated in the presence and absence of 80-mer ssDNA. N. Calcium

transient time to decay 25% for isoproterenol treated or untreated in the presence and absence of 80-mer ssDNA. All results are expressed as mean $\pm$ SEM. Multi-group comparisons were assessed using one way analysis of variance (ANOVA) with Bonnferoni post-hoc test with * $P < 0.05$ considered statistically significant.

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Figure 2.

Multiple XNA moieties of completely random sequence 50-mers enhance relaxation of adult cardiac myocytes. **A.** Representative structures of the XNAs tested showing the chemical modification for each. **B.** Time to return to 25% baseline sarcomere length from peak amplitude of contraction as a measure of relaxation for 40-mer ssDNA (DNA) the complement strand 40-mer ssDNA (Comp DNA) and double stranded DNA (dsDNA) transfected cardiac myocytes. **C.** Time to return to 25% baseline sarcomere length from peak amplitude of contraction as a measure of relaxation for 50-mer random sequence ssDNA (DNA) and L-DNA (enantiomer). **D.** Time to return to 25% baseline sarcomere length from peak amplitude of contraction as a measure of relaxation for 50-mer random sequence ssDNA (DNA), 2'-o-methyl RNA (2-OMe) and phosphorothioate backbone DNA (PS). All results are expressed as mean $\pm$ SEM. Multi-group comparisons were assessed using one way analysis of variance (ANOVA) with Bonferroni post-hoc test with * $P < 0.05$ significantly different from control no DNA, ** different from all groups.

Figure 3.

Protein translation and innate immune responses are not necessary for enhanced relaxation of XNA in adult cardiac myocytes. **A.** Timeline of experimental procedure for cycloheximide (CHX) treatment. Cells were treated with 100 μ M CHX for 10 minutes and then transfected with 50-mer random sequence ssDNA in the presence of CHX. **B.** Sarcomere dynamics measure of peak amplitude of contraction and time to return to 25% baseline sarcomere length $\pm$ CHX and $\pm$ 50-mer ssDNA. **C.** Representative structure of Poly-T ssDNA with 50 thymine nucleotides. Sarcomere dynamics measurements of relaxation, time to BL 25% for random sequence 50-mer ssDNA (50 DNA) or 50-mer all thymine nucleotides. All results are expressed as mean $\pm$ SEM. Multi-group comparisons were assessed using one way analysis of variance (ANOVA) with Bonferroni post-hoc test with * $P < 0.05$, significantly different from control no DNA, # or ** different from all groups.

Figure 4.

Sarcomeric proteins bind random sequence ssDNA with high affinity. A. Fluorescence polarization binding curves for phospholamban (PLN), myosin S1 and bovine cardiac troponin complex (Troponin) with 50-mer random sequence ssDNA. B. Fluorescence polarization binding curves for myosin S1 and bovine cardiac troponin complex (Troponin) with 50-mer double-stranded DNA. C. Affinity capillary electrophoresis (ACE) and fluorescence polarization (FP) determined dissociation constants for human cardiac troponin complex (Complex) and the individual subunits of the complex: cardiac troponin I (TnI), cardiac troponin T (TnT) and cardiac troponin C (TnC). D. XNA-Sarcomere binding assay, top panel α -actinin (green), second panel AF-594 40-mer (red), third panel overlay with DAPI (blue), fourth panel blowup of inset box in overlay.