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Specific inhibition of Na–Ca exchange function by antisense oligodeoxynucleotides

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Abstract **The Na–Ca exchanger is essential for the Ca^{2+} homeostasis in many cell types. This transporter has been difficult to investigate because no specific inhibitor is available. We have synthesized an antisense oligodeoxynucleotide directed against the rat cardiac Na–Ca exchanger mRNA. To estimate the activity of the Na–Ca exchange in single cultured myocytes, the exchange current (I_{NaCa}) was measured with the voltage-clamp technique while the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was simultaneously recorded. Most cells exposed to antisense oligodeoxynucleotide showed neither an I_{NaCa} nor an increase of $[Ca^{2+}]_i$ upon extracellular Na^+ removal. Liberation of Ca^{2+} by flash-photolysis of caged Ca^{2+} was not followed by a decay of $[Ca^{2+}]_i$ in cells exposed to the antisense oligonucleotide, whereas in control cells resting $[Ca^{2+}]_i$ was reached 6 s after the flash. Control experiments with non-sense and mismatched oligonucleotides were performed to exclude unspecific inhibitory effects. These results demonstrate that the Na–Ca exchange was specifically and completely suppressed and that antisense oligodeoxynucleotides represent a useful tool to investigate the cellular and molecular properties of the Na–Ca exchanger.**

Key words: Na–Ca exchanger; Antisense oligodeoxynucleotide; Confocal microscopy; Flash photolysis; Caged compound; Ca^{2+} measurement

1. Introduction

The Na–Ca exchanger is a transport protein located in the membrane of almost every cell type. It usually performs uphill transport of Ca^{2+} with a stoichiometry of 3 Na^+:1 Ca^{2+} using the electrochemical Na^+-gradient, thus generating a membrane current. In cardiac muscle cells it is well established that the Na–Ca exchange is responsible for removal of Ca^{2+} entering the cell via the L-type Ca^{2+} current to trigger Ca^{2+} release from the SR (Ca^{2+} induced Ca^{2+} release, CICR [1,2]). More recently, a much faster role for the Na–Ca exchange has also been suggested based on the observation that under some conditions Na^+ current can also trigger CICR, presumably via 'backward' Na–Ca exchange (i.e. the Ca^{2+} influx mode [3,4], but see also [5,6]).

Experimentally, the Na–Ca exchange activity has been measured in various ways in cardiac myocytes and other preparations. Either as a *trans* Na^+-dependent *cis* Ca^{2+}-activated membrane current or as a Ni^{2+} sensitive membrane current [7,8]. Some of these studies were combined with experiments in which the transport function of the Na–Ca exchange was assessed by following changes in $[Ca^{2+}]_i$ with fluorescent indicators [9,10].

Although the cellular function and the steady-state kinetics of this transporter have been characterized in some detail, very little is known about the molecular function. This is in part due to the fact that there is still no specific pharmacological inhibitor available for the Na–Ca exchange. After the exchanger had been cloned and sequenced, it was generally expected that molecular biology techniques, in combination with the biophysical methods mentioned above, may shed some light on the molecular operations of the Na–Ca exchanger [11,12]. Meanwhile, a number of Na–Ca exchanger isoforms has been identified in different tissues [13] and several mutants have been genetically engineered, some indeed with interesting functional modifications [14].

Here we present results obtained with a complementary approach. We designed antisense oligodeoxynucleotides (AS-ODNs) directed against the Na–Ca exchanger mRNA of rat cardiac myocytes. Exposure of cultured neonatal myocytes to the ODNs resulted in a rapid (≈24 h) suppression of Na–Ca exchange activity, as revealed by measurements of Ca^{2+} transport and membrane currents generated by the electrogenic Na–Ca exchange.

The comparison of cells expressing very low levels of Na–Ca exchanger with control preparations may not only provide additional insight into the cellular function of this protein, but may also allow the identification of molecular functional details of the transporter. Preliminary results have been presented to the Biophysical Society [15].

2. Materials and methods

2.1. Ca^{2+} measurements

Intracellular Ca^{2+} was measured ratiometrically using a confocal microscope and a mixture of two fluorescent Ca^{2+} indicators. The cells were dialyzed with the salt-form of fluo-3 and fura-red in the whole-cell recording mode of the patch-clamp technique. In addition to image sequences rapid line-scans were performed with the laser-scanning confocal microscope (MRC600; Bio-Rad, Glattbrugg, Switzerland) to obtain the Ca^{2+} concentration with a high temporal resolution (up to 500 Hz). Absolute Ca^{2+} concentrations were calculated from the fluorescence signals using an in vivo calibration curve. The setup as well as the ratiometric technique have been described in detail [16]. Measurements of Ca^{2+} concentration changes are shown as mean ± S.E.M. in the figures. Significant differences, as determined by a paired Student's *t*-test ($P < 0.05$) are indicated by an asterisk.

2.2. Experimental solutions

The superfusion solution contained (in mM): NaCl 140, KCl 5, $CaCl_2$ 2, HEPES 10, glucose 10. The pH was adjusted to 7.4 with NaOH. Extracellular solutions were changed with a $t_{1/2} < 0.5$ s using a multi-barelled rapid switching system. Li^+ was used as a substitute in Na^+_o-free solutions. Ryanodine 10 μM and thapsigargin 0.2 μM were added

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30 min prior to an experiment to inhibit the sarcoplasmic reticulum and to prevent interference from this Ca^{2+} store with the Ca^{2+} extrusion by the Na–Ca exchange. The pipette filling solution contained (in mM): Cs-aspartate 120, NaCl 10, K-ATP 4, Tetraethylammonium-Cl 20, HEPES 10, fluo-3 0.033, fura-red 0.066 (Molecular Probes, Eugene, OR, USA). For some experiments, Na_4-DM-nitrophen 2, reduced glutathione (GSH) 2 and Ca^{2+} 0.5 were added to the pipette filling solutions. The pH was adjusted to 7.2 with CsOH. All experiments were carried out at room temperature (20–22°C).

2.3. Experimental protocol

Cells exposed to the ODNs and control cells were voltage-clamped and dialyzed with the fluorescent Ca^{2+} indicators. After establishing whole-cell recording conditions sodium currents (I_{Na}) were activated to verify viability and the cardiac muscular origin of each tested cell. The Na–Ca exchange was subsequently activated in the Ca^{2+} influx mode ('backward-mode') by reversing the Na^+-gradient, i.e. by rapid superfusion with a Li^+ containing Na_o^+-free solution. In cells exhibiting Na–Ca exchange this resulted in an increase of $[Ca^{2+}]_i$. Upon readdition of Na_o^+, Ca^{2+} removal from the cell by the Na–Ca exchange was initiated. In cells with very little remaining exchange activity, the Na -free solution did not induce detectable Ca^{2+} loading. Such cells were additionally dialyzed with the caged Ca^{2+} compound DM-nitrophen. Ca^{2+} concentration jumps were then generated by photolyzing the caged compound with a short flash of intense UV-light (duration ≈ 1 ms, discharged energy 100 Ws) [8] while simultaneously measuring $[Ca^{2+}]_i$ and I_{NaCa}.

2.4. Cell culture

Primary cultures of neonatal rat cardiac myocytes were prepared using established methods [17]. Briefly, cardiac myocytes were enzymatically isolated and cultured from 2-days old Wistar rats. A drop of cell suspension was placed on a round cover slip in a Petri-dish. The AS-ODN was added at an initial concentration of 3 μM after 2 h. Control cultures were established in parallel. The Petri-dishes were stored at 37°C in a 1.5% CO_2 incubator until use (12–72 h).

2.5. Synthesis and purification of phosphorothioate oligodeoxynucleotides (ODNs)

Phosphorothioate ODNs were synthesized on a Geneassembler A 470 (Pharmacia, Uppsala, Sweden) using standard phosphoramidite chemistry and the specific thiolating reagent. The ODNs were either purified on a FPLC system (Pharmacia, Uppsala, Sweden) using a Mono Q anion exchange column or simply desalted by passing through NAP-5 columns (Pharmacia). The nucleotides were concentrated in a Speed-Vac and stock solutions of purified ODNs (100–300 μM) were stored at 4°C or for longer periods at −70°C.

3. Results

3.1. Design of phosphorothioate oligodeoxynucleotides (ODNs)

In several cases it has been reported that the most effective antisense ODNs were targeted to the 3′ untranslated region (3′ UTR) of the RNA [18,19]. For these reasons we have chosen a sequence in the 3′ untranslated region of the cardiac Na–Ca exchanger which is 100% conserved in man, rat and dog. The sequences of the antisense (AS), the non-sense (NS, reverse 5′ to 3′ orientation compared to antisense) and the mismatched (MM, containing 3 mismatches compared to the AS-ODN) oligodeoxynucleotides are listed in Fig. 1.

3.2. Antisense oligodeoxynucleotides reduce Na–Ca exchange function

For the functional studies cultures of control cells and cultures exposed to the ODN (3 μM added at time zero) were established in parallel. Several techniques were used to specifically assess the Na–Ca exchange activity. First, the Na^+ concentration gradient across the sarcolemma of voltage-clamped cells was reversed by replacing extracellular Na^+ with Li^+ using a rapid solution switcher ($t_{1/2} < 0.5$ s). In the presence of extracellular Ca^{2+} this maneuver is expected to induce Ca^{2+} entry into the cells via the 'reverse mode' of the Na–Ca exchange. The activity of the exchange in the reverse mode was estimated by determining the initial rate of increase in intracellular Ca^{2+} (V_{up}) measured with ratiometric confocal microscopy in the line-scan mode. All experiments were performed in the continuous presence of ryanodine and thapsigargin to eliminate interference from the sarcoplasmic reticulum. In the presence of these blockers and in the time frame analyzed, Ca^{2+} entry and removal from the cytosol were almost exclusively due to Na–Ca exchange as suggested by the absence of the $[Ca^{2+}]_i$ decay when the Na–Ca exchange was completely suppressed (see below).

Panel A of Fig. 2 shows representative Ca^{2+} signals recorded in a control cell (upper trace) and in an exposed cells (lower trace). During the Na_o^+-free period indicated by a horizontal line $[Ca^{2+}]_i$ rose by about 3 μM. Immediately after the Na_o^+-free period Na_o^+ was rapidly reapplied to activate the Na–Ca exchange in the Ca^{2+} extrusion or 'forward' mode. This intervention led to a monotonic decay of $[Ca^{2+}]_i$ to resting values. The τ of this process was evaluated to determine the activity of the Na–Ca exchange as a Ca^{2+} removal system (i.e. the 'forward mode'). In the cell exposed to 3 μM of the antisense ODN for 24 h the same experiment resulted in a much slower and smaller increase of $[Ca^{2+}]_i$, suggesting that this cell exhibited significantly less Na–Ca exchange activity.

The onset of the inhibition by antisense ODNs is assumed to reflect the degradation and turnover of the Na–Ca exchanger proteins in the cell membrane. We performed the functional studies 24 h and 48 h after adding 3 μM of the nucleotide. In control cells, the mean V_{up} was 350 ± 35 nM/s as determined during the first two seconds in zero $[Na^+]_o$. However, in exposed cells, V_{up} was only 21.8% of control after 24 h and 3.8% after 48 h, respectively (see panel B of Fig. 2 for a summary of normalized data). Note that after 48 h 5 out of 7 cells exhibited no detectable increase in Ca^{2+} while the two other cells had a

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                  3051                                          3090
RAT               TGAACAGAGG AAACTGACAT TTGTCATGTT CACTTAACCT
HUMAN             TGGACAGAGG AAACTGACAT TTGTCATGTT CACTT.ACCT
DOG               TGAACAGAGG AAACTGACAT TTGTCATGTT CACTT.ACCT

Antisense ODN  3'              ACTGTA AACAGTACAA GTG        5'
Non-sense ODN  5'              ACTGTA AACAGTACAA GTG        3'
Mismatched ODN 3'              AATGTA CACAGTACAA TTG        5'
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Fig. 1. Nucleotide sequence comparison of parts of the 3′ untranslated region of the cardiac Na–Ca exchanger cDNA (numbering according to the rat sequence [31] and the ODNs used in the experiments. The mRNA region hybridizing with the phosphorothioate antisense oligodeoxynucleotide (bold) is 100% conserved in all 3 species. The 3 mismatches introduced in the mismatched ODN are underlined.

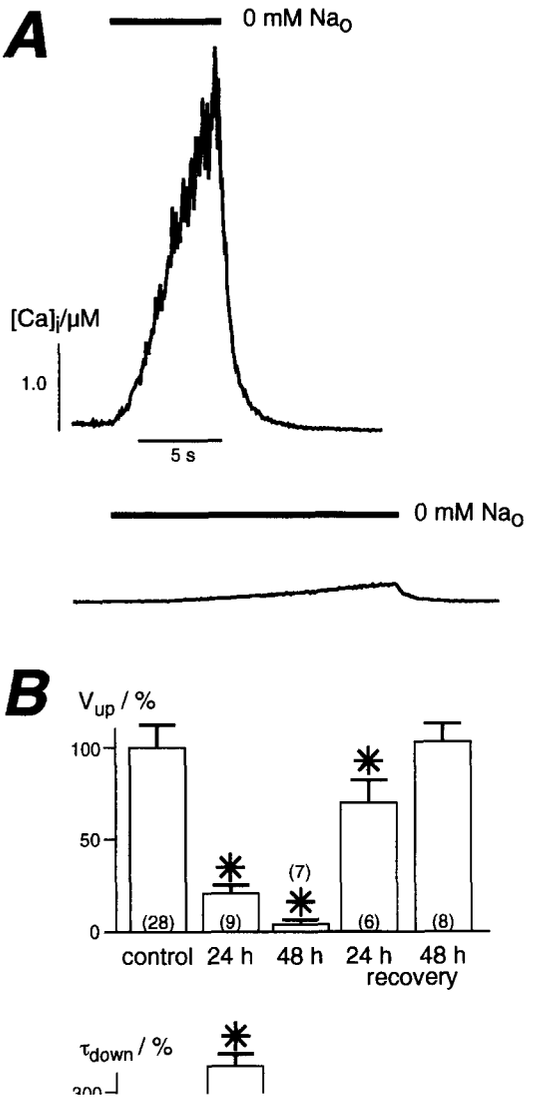

Fig. 2. Suppression of Na–Ca exchange by exposure to 3 μM antisense ODN. (A) Shows representative Ca^{2+}-transients elicited by removal of Na_o^+ in a control cell (upper trace) and in a cell exposed for 24 h (lower trace). Cultured neonatal rat ventricular myocytes were patch-clamped and held at −50 mV. Normalized initial Ca^{2+} increase rates (V_{up}) and the decay of the Ca^{2+}-transients (τ_{down}) are summarized in (B) for control cells (pooled data) and cells exposed for 24 or 48 h. The histograms also show the recovery of the Na–Ca exchange function after removal of the ODN (following 24 h exposure). Note that after 48 h τ_{down} was not determined because 5 out of 7 tested cells showed no increase of $[Ca^{2+}]_i$. An asterisk denotes a significant inhibition compared to control ($P<0.05$).

very low Na–Ca exchange activity. The statistics also show that following an incubation period of 24 h the V_{up} recovers to 71.3% 24 h and to 103.4% 48 h after removal of the ODN from the culture medium. The lower histogram of Fig. 2B allows a comparison of the decay of the Ca^{2+}-transient (τ_{down}) under different conditions. As expected, the effects of Na–Ca exchange inhibition on τ_{down} paralleled those on V_{up}.

As a consequence of the almost complete inhibition of the Na–Ca exchange after 48 h, these cells could not be loaded with Ca^{2+} by Na_o^+ removal and thus the Ca^{2+} efflux mode of the exchanger could not be analyzed with this approach without introducing a serious statistical bias (i.e. only the cells with incomplete inhibition of the Na–Ca exchange would have been evaluated). Therefore, we had to resort to another technique to elevate $[Ca^{2+}]_i$. Intracellular Ca^{2+} concentration-jumps were performed with UV-flash photolysis of caged Ca^{2+}. For this purpose, single cells were dialyzed with a pipette filling solution containing 2 mM DM-nitrophen and 0.5 mM Ca^{2+} (in addition to the Ca^{2+} indicators fluo-3 and fura-red). During these experiments, a confocal image was recorded every two seconds together with the membrane currents and the results are shown in Fig. 3A for a control cell and in Fig. 3B for a cell exposed to the antisense ODN for 48 h. While superfusion with zero $[Na^+]_o$ resulted in an increase of $[Ca^{2+}]_i$ to about 1.8 μM in the control cell, this maneuver had almost no effect in the exposed cell, indicating that virtually no Na–Ca exchange activity was left. Upon an intense flash of UV-light, $[Ca^{2+}]_i$ jumped to 1.5 μM in the control cell and then decayed to resting values within 6 seconds. In the exposed cell without significant Na–Ca exchange function upon Na_o^+ removal, the flash resulted in a comparable Ca^{2+} concentration jump, but there was essentially no decay of the Ca^{2+} concentration over the time examined, suggesting (i) that the Na–Ca exchange was almost completely blocked and that (ii) all other Ca^{2+} transport systems are not

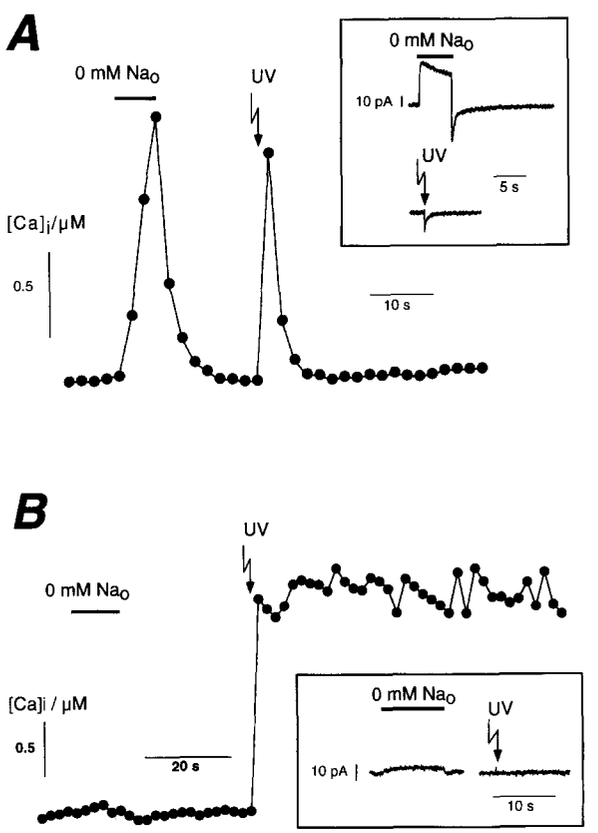

Fig. 3. Intracellular $[Ca^{2+}]$ and membrane currents after 48 h exposure. (A) Illustrates data obtained from a control cell. Each data point (●) reflects the average cytosolic $[Ca^{2+}]$ determined in a ratiometric confocal image. Frames were acquired at 0.5 Hz. Removing extracellular Na^+ for 10 seconds and flash-photolysis of caged Ca^{2+} resulted in transient increases of $[Ca^{2+}]_i$. Corresponding changes of Na–Ca exchange outward and inward current are shown in the inset. In the exposed cell Na_o^+ removal did not change $[Ca^{2+}]_i$ significantly (B) and the flash photolytic Ca^{2+} concentration jump did not decay during the recording. In addition, no Na–Ca exchange currents could be detected.

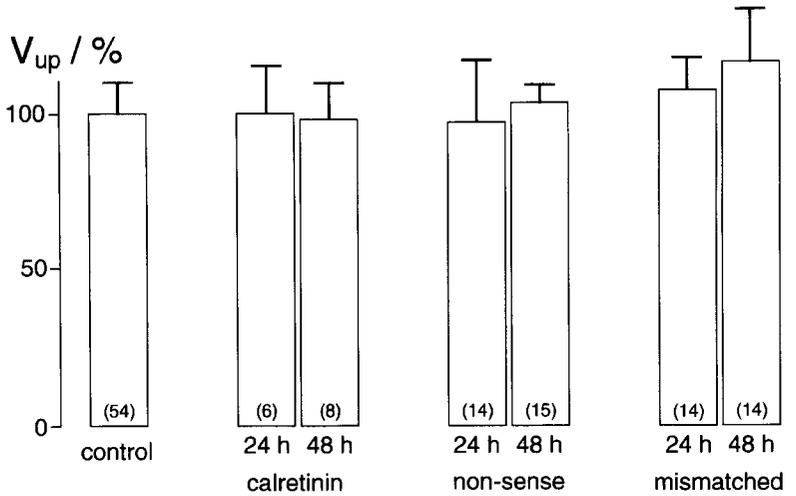

Fig. 4. Unexposed cells were compared to cells exposed to 3 μM control ODNs. Normalized V_{up} values were determined in cells exposed to an AS-ODN directed against calretinin, a non-sense ODN and a mismatched AS-ODN. No significant inhibition was found for any of the control sequences after 24 or 48 h.

significantly removing Ca^{2+} under these conditions (i.e. in the presence of ryanodine and thapsigargin).

Since the Na–Ca exchanger is an electrogenic transporter with a stoichiometry of 3 Na^+:1 Ca^{2+}, an outward membrane current is expected to be generated in the Ca^{2+} influx mode, while an inward current is produced in the Ca^{2+} efflux mode [8,20]. The membrane-current changes during and after Na_o^+ removal and after photorelease of Ca^{2+} are also shown in Fig. 3. During Na_o^+ removal, there was an outward current exhibiting a slow decay in the control cell (panel A). Although we have not further characterized this current, a significant fraction was due to Na–Ca exchange electrogenicity, since it was essentially absent in the exposed cell. The small and steady outward shift of membrane current during the absence of Na_o^+ in the exposed cell was presumably due to some difference in the membrane background conductance for Na^+ and Li^+. After both, Na_o^+ readdition and the UV-flash a significant inward current was activated in the control cell (panel A), reflecting Ca^{2+} removal by the Na–Ca exchange [8]. The inward current was transient and decayed as intracellular Ca^{2+} was reduced by the exchanger itself. Neither Na_o^+ readdition nor flash-photolysis of caged Ca^{2+} generated a detectable transient inward current in the exposed cell. The dramatic reduction of the membrane currents again indicates that the Na–Ca exchange activity was almost completely suppressed in the cell initially exposed to 3 μM of the antisense ODN.

3.3. *Control experiments with non-sense and mismatched oligodeoxynucleotides*

We performed several series of control experiments to exclude unspecific direct or indirect effects of the nucleotide on the Na–Ca exchange. For this reason a minimum amount of control experiments have been suggested by several authors [21,22]. In our approach 3 control oligos were used: (i) a non-sense ODN having a reversed 5′ to 3′ orientation compared to the antisense ODN, but maintaining the same base composition; (ii) a mismatched (MM) ODN containing 3 mismatches compared to the antisense ODN (as listed in Fig. 1) which should not efficiently anneal to the target sequence, since the annealing temperature T_m is too low; (iii) an ODN directed against the calcium-binding calretinin [23] which is not expressed in cardiac cells. All of these control ODNs should not suppress Na–Ca exchange function significantly. Furthermore, we used phosphorothioate oligodeoxynucleotides, the best characterized class of oligodeoxynucleotide derivatives, because they can be added directly to the culture medium and are more resistant to endo- and exonucleases than phosphodiester oligodeoxynucleotides [24]. An additional advantage of the phosphorothioate oligodeoxynucleotides is their high affinity for RNA targets and also the activation of RNase H. These oligodeoxynucleotides can often be applied in 10-fold lower concentrations than regular oligodeoxynucleotides thus diminishing non-specific cytotoxic effects [18]. Cells from cultures exposed to each of these control nucleotides for 24 and 48 h were compared with control cells from parallel cultures. All tested control nucleotides had no significant inhibitory effect on the Na–Ca exchange activity as summarized in Fig. 4, suggesting that the inhibition observed in the presence of the antisense ODN resulted from an interaction specific for this sequence.

4. Discussion

The results presented in this study demonstrate that the engineered antisense ODN can rapidly (within less than 48 h) and completely remove the Na–Ca exchange activity in cultured rat cardiac myocytes as assessed by Ca^{2+} transport and membrane current measurements. This approach therefore enabled us to inhibit the Na–Ca exchange selectively, and can be applied to identify and characterize cellular and molecular features of Na–Ca exchange function. For example, the membrane current generated by the Na–Ca exchange (I_{NaCa}) has so far only been identified indirectly by evaluating the voltage-dependence of Ca^{2+} induced membrane currents or by investigating the effects of counter-ion removal. All available inhibitors turned out to be unspecific (e.g. Ni^{2+}, La^{3+}, DCB) with the possible exception of the exchanger inhibitory peptide (XIP), which only inhibits from the inside of the cell [25].

Up to now, none of the presently used unspecific inhibitors has an identified mode of action on the molecular level. It is not known a priori, which of the molecular reaction steps in the Na–Ca exchange cycle is inhibited and which molecular transitions are still possible in the presence of a particular inhibitor. Since the AS-ODNs are aimed to inhibit the de novo synthesis of a given protein, after a certain lag period depending on the half-life of the protein and the kinetics of the ODN uptake, the Na–Ca exchanger should be efficiently eliminated from the sarcolemma. Therefore, any biological signals associated with the Na–Ca exchanger should be greatly diminished or even be absent. In combination with the known unspecific inhibitors mentioned above the technique may thus help to identify molecular functional features of both the Na–Ca exchanger and some of the inhibitors. A similar technique has been successfully applied to measure the kinetics of molecular conformational changes of the Na–K-ATPase. These experiments have been possible because specific inhibitors for this transporter are readily available [26,27].

The changes of Ca^{2+} signaling after suppression of the Na–Ca exchanger observed in the present study were also dramatic. The increase of $[Ca^{2+}]_i$ during removal of extracellular Na^+ was completely absent in most exposed cells, suggesting that there was virtually no Na–Ca exchange activity left. With the combined presence of ryanodine and thapsigargin we expected to completely inhibit the SR since we already showed that each compound alone was able to inhibit Ca^{2+}-induced as well as caffeine-induced Ca^{2+} release in the same preparation [28]. Exposure to the ODN would thus leave the cells only with the sarcolemmal Ca^{2+} pump and the mitochondria as known Ca^{2+} uptake and extrusion systems. The transport capacity of these two systems has recently been estimated in adult rat cardiac myocytes by following the relaxation during inhibition of the SR Ca^{2+} pump and the Na–Ca exchange. In these experiments relaxation was very slow and it was deduced that the contribution of the mitochondria and sarcolemmal Ca^{2+} pump to Ca^{2+} removal was only about 1% [29]. These conclusions are consistent with our observation that in cells without residual Na–Ca exchange activity the flash photolytic elevation of intracellular Ca^{2+} did not decline during the time examined (compare Fig. 3A with Fig. 3B), indicating that the contribution of the sarcolemmal Ca^{2+} pump and the mitochondria was not relevant over the time frame examined.

In addition to its important role during Ca^{2+} removal and relaxation the Na–Ca exchange has recently been implicated in more rapid events during cardiac excitation contraction coupling. In several studies, Na^+ currents have been found to trigger SR Ca^{2+} release (for example [3,4]), presumably by activating the Na–Ca exchange in the Ca^{2+} influx mode after Na^+ accumulation in a restricted space under the sarcolemma [30]. But the physiological role of this signaling pathway is under current debate and its relevance is not generally accepted [5,6]. Antisense ODNs directed against the Na–Ca exchanger may represent a useful and specific tool to analyze this signaling pathway in cardiac muscle as well as the cellular role of the Na–Ca exchange in various cell types relying on cellular Ca^{2+} signaling mechanisms.

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