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Effects of Antisense Oligonucleotides to the Cardiac Na^+/Ca^{2+} Exchanger on Calcium Dynamics in Cultured Cardiac Myocytes

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The present study was designed to explore the role of the Na^+/Ca^{2+} exchanger on spontaneous beating of cultured cardiac myocytes. Antisense oligonucleotides (AS) based on the sequence of the cardiac Na^+/Ca^{2+} exchanger were used to decrease expression of this Ca^{2+} transporting protein in cardiac myocytes. An application of AS (10 μM) caused an increase in beating rate of myocytes within 6–24 h. After 24 h of exposure, AS increased the beating rate from an average rate of 77 beats/min in control and sense-treated myocytes to 103 beats/min. Moreover, myocytes treated for 24 h with 10 μM AS exhibited an increase in diastolic $[Ca^{2+}]_i$ levels. The antisense treatment also led to a ~20% decrease in expression of Na^+/Ca^{2+} exchanger proteins within 6–24 h. Changes in mRNA levels following AS treatment could not be detected within 3- to 24-h periods. The results of these studies suggest that the Na^+/Ca^{2+} exchanger plays a potentiating role in spontaneous the beating process by regulating $[Ca^{2+}]_i$ dynamics and that even a small reduction in the levels of the exchanger protein has marked effects on the handling of $[Ca^{2+}]_i$ during the cardiac cycle. © 1999 Academic Press

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The cardiac sarcolemmal Na^+/Ca^{2+} exchanger and the plasma membrane Ca^{2+}-ATPase are the two plasma membrane Ca^{2+} transporting proteins responsible for the extrusion of excess intracellular Ca^{2+} between and during cycles of excitation-contraction coupling (1–3). Although it has been proposed that the exchanger also operates in the reverse mode to bring Ca^{2+} into the cells, there has been controversy regarding the participation of the exchanger in Ca^{2+} influx and stimulation of further Ca^{2+} release from the sarcoplasmic reticulum (SR) (4–6). The lack of highly specific inhibitors of the exchanger has hampered resolution of this issue. Detailed studies of the coupling between Ca^{2+} channels and ryanodine receptors in producing the rise in $[Ca^{2+}]_i$ that leads to contraction have suggested that the exchanger does not substantively contribute to the Ca^{2+} influx and that it may actually be seregated from the Ca^{2+} microdomain in which the dihydropyridine-sensitive channel and the ryanodine receptor reside (5–7). The acquisition of significant information about the molecular structure of the various isoforms of the exchanger proteins has provided another strategy through which the physiological role of the Na^+/Ca^{2+} exchanger can be probed by manipulating levels of the protein expressed in cells in culture and *in vivo.* Lipp and colleagues (8) used an antisense (AS) oligonucleotide to eliminate the exchanger-mediated current (I_{Na-Ca}) in single cardiac myocytes, and Slodzinski *et al.* (9) used AS oligo to reduce exchanger expression in arterial myocytes and showed elevated resting intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) but unaltered responses to serotonin. We have also previously used AS oligos to examine the effects of decreasing exchanger expression on resting $[Ca^{2+}]_i$ and the beating rate of primary cultures of cardiac myocytes (10). And, more recently, cardiac myocytes from transgenic mice overexpressing the Na^+/Ca^{2+} exchanger have been used to address the question of the primary physiological role that the exchanger plays in regulating $[Ca^{2+}]_i$ during the cardiac cycle (11–13).

The use of an AS probe to conserved region in all known isoforms of the exchanger led to an elevation in resting $[Ca^{2+}]_i$ and a substantial increase in the beating rate in primary cardiac myocytes derived from em-

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bryonic rat pups (8). These observations suggested that the reduced expression of the exchanger protein primarily affected Ca^{2+} efflux activity in the cells, leading to an elevation in cytosolic Ca^{2+} and the more frequent triggering of contractions. In our earlier studies (10) the exchanger activity appeared to be reduced by about 30% at 24 h following exposure to the AS oligo, though the relative levels of exchanger protein expression were not determined. The goals of the present study were (i) to determine whether the AS treatment induced detectable decreases in expression of the exchanger mRNA, protein or both, and (ii) to determine whether the AS treatment led to alterations in the free $[Ca^{2+}]_i$ associated with the diastolic and the systolic components of the cardiac cycle.

MATERIALS AND METHODS

Preparation of cardiac myocyte culture. Preparation of primary cardiac myocyte and nonmyocyte cultures from 1-day-old Wistar rats was carried out as previously (14). For selective enrichment with cardiac myocytes, dissociated cells were preplated for 1 h, during which period the nonmyocytes attached readily to the bottom of the culture dish. Non-adherent cells, mostly myocytes, were plated at a density of $2–5 \times 10^6$ cells/ml/dish. Bromodeoxyuridine (0.1 mM) was added during the first 2 days to prevent proliferation of the nonmyocytes. This procedure yielded cultures with 90–95% myocytes, as assessed by microscopic observation of cell beating (10). Cells were kept in serum-containing culture medium [Dulbecco's modified Eagle's medium/F-12 (GIBCO) (1:1, v/v) supplemented with newborn calf serum (5%), 3 mM pyruvic acid, 100 μM ascorbic acid, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium:DMEM/F-12 medium] for 48 h.

Oligonucleotide treatment of myocyte cultures. The synthetic oligonucleotides used here were those used previously with rat embryonic myocytes (10) and had the following sequences: 5′-AGGAACACGTTCACGGCGTT-3′ (AS) and 5′-AACGCCGTGAACGTGTTGCT-3′ (S). The sequences showed no homology to any DNA in the GenBank except Na^+/Ca^{2+} exchangers. The AS oligonucleotide is complementary to a region near the 3′ end of the canine heart exchanger mRNA transcript (nucleotides 2638–2657) (10). This series of nucleotides is totally conserved in all three known isoforms of exchanger (15–17). This sequence encodes the peptide NAVNVFL, presumed to be located in the ninth transmembrane domain of the three isoforms. The S and AS oligonucleotides were dissolved in Lipofectamine (Gibco BRL) at a ratio of DNA:Lipofectamine of 10:1. The cells were exposed to oligonucleotides (10 μM) in serum-free medium for 1 h, then serum was added to the final concentration of 5% and incubation continued for 23 h. Control dishes received appropriate amounts of Lipofectamine only.

Analysis of RNA and exchanger protein expression. Total cellular RNA of cardiac cells was isolated using the acid guanidinium thiocyanate-phenol–chloroform extraction method (18). Northern blot analysis was performed according to the procedure described by Kim *et al.* (19) and represented three independent experiments. The following probes were used for Northern blot analysis: (1) Na^+/Ca^{2+} exchanger: C-terminus; 1.3-kb *Eco*RI fragment of the rat Na^+/Ca^{2+} exchanger cDNA and (2) glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 1.3-kb *Pst*I/*Pst*I fragment of rat GAPDH cDNA (a gift from S. Kim and H. Iwao, Osaka City University, Medical School).

The effect of treatment with the oligos on expression of the exchanger protein was determined by ELISA analysis performed according to the procedures and conditions described previously (20). Samples (2 μg protein in 100 μl) of cardiac myocytes exposed to the various treatments were fixed to the wells of ELISA plates by addition of 100 μl of 0.5% (v/v) glyceraldehyde in PBS. Following the blocking of non-specific sites with 100 mM glycine-2% (w/v) in PBS, antiserum against the cardiac Na^+/Ca^{2+} exchanger (Swount, Bellincona Switzerland) was added at a dilution of 1:200 in PBS-0.05% Tween 20 and incubated for 2 h at room temperature. The second antibody, a goat anti-rabbit IgG alkaline phosphatase conjugate (Zymed Laboratories) was added at a dilution of 1:1000 and incubated for 1 h at room temperature. After extensive washing of the plates, the substrate p-nitrophenyl phosphate (1 mg/ml) in Tris-buffer, pH 9.8, was added. Following a 30-min incubation in the dark, the absorbance of reaction product was determined at 405 nm. The protein concentration in the myocyte samples was determined using the bicinchoninic acid assay (Pierre Chemical Co. Rockford, IL).

Measurement of $[Ca^{2+}]_i$ and beating. The method of measuring of $[Ca^{2+}]_i$ has been described by Takahashi *et al.* (14). In brief, fura-2 loading was performed by the addition of fura-2/AM (2 μM dissolved in dimethyl sulfoxide with 0.1% cremophor EL) into a Petri dish containing 1 ml of culture medium and glass coverslips on which cells were attached. After mixing, the dishes were incubated in the dark for 30 min in humidified 5% CO_2–95% air atmosphere at 37°C. The medium was removed, and replaced with fresh culture medium. A coverslip with fura-2 loaded cells was placed in an experimental chamber of 37 ± 0.5°C. The cells were incubated in DMEM/F-12 medium containing with 10 mM N-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid (Hepes) (pH 7.4) and 5% serum. An INTERDEC #M-1000 fluorescent microscope system (Osaka, Japan) was used to evaluate $[Ca^{2+}]_i$. The results are generally presented as fura-2 ratios. Background fluorescence was determined using nonlabeled cells and was subtracted from the data obtained with fura-2 loaded cells. No dye leakage from the cells could be detected 1 h after initiating the experiments. During the course of one cycle of contraction and relaxation, the 340/380 fluorescence ratio increased to a maximum value during systole, and fell to a minimum value during diastole as illustrated in Fig. 1. The calcium transient (Ca-T) represents the difference between the Max and Min values. All data are expressed as Max, Min, and Ca-T and represent the mean of 10 beats.

The beating status of cultured cardiac myocytes was monitored at 37°C with an inverted phase-contrast microscope at magnifications of 100 to 200×. Beating rates of individual cells were counted over periods of 20–60 s as previously reported (21).

Statistics. Statistical significance was determined by χ^2-test, Student's t-test or analysis of variance (ANOVA). Bonferroni's method was used to compare individual data when a significant *F* value was shown depending on the design of the experiments. Each value was expressed as the mean ± SEM. Differences were considered statistically significant when the calculated *P* value was less than 0.05.

RESULTS

Effects of the Na^+/Ca^{2+} Exchanger AS Oligonucleotide on Protein Expression and Beating of Cardiac Myocytes

Exposure of the cardiac myocytes to the AS oligo nucleotide decreased expression of the protein and increased myocyte beating rates in a time-dependent manner (Table 1). The time course for decreasing Na^+/Ca^{2+} exchanger expression following application of the AS oligonucleotide revealed that the exchanger expression significantly decreased by 6 h and reached a maximal reduction of 20% at 24 h following treatment (Table 1). The control myocytes formed monolayer sheets of synchronously beating cells, whose rate of

TABLE 1

Time Course of Antisense Oligonucleotide Suppression on Na^+/Ca^{2+} Exchanger Proteins in Neonatal Cardiac Myocytes

Time (h)	Exchanger protein (Absorbance, 405 nm)	Beating rates [beats/min (% of initial)]
0	0.372 ± 0.018 (20)	136 ± 7 (35) (100%)
3	—	129 ± 13 (29) (95%)
6	0.321* ± 0.014 (19)	240# ± 7 (19) (176%)
12	0.316* ± 0.013 (19)	234# ± 24 (15) (172%)
24	0.298** ± 0.011 (19)	—

Note. The cells were treated with 10 μM antisense oligonucleotide in Lipofectamine for the indicated time. ELISAs were used to estimate the relative levels of exchanger proteins in the samples, and the data are presented as mean (±SEM) absorbance values from three experiments with more than six determinations for each condition. The absorbance values for cells treated with the S-oligo for 12 h were 0.351 ± 0.017 (n = 15). Each value of beating rate represents the mean ± SEM (n = 19–35). Asterisks indicate significant differences from 0 h: *$P < 0.05$, **$P < 0.01$, #$P < 0.001$.

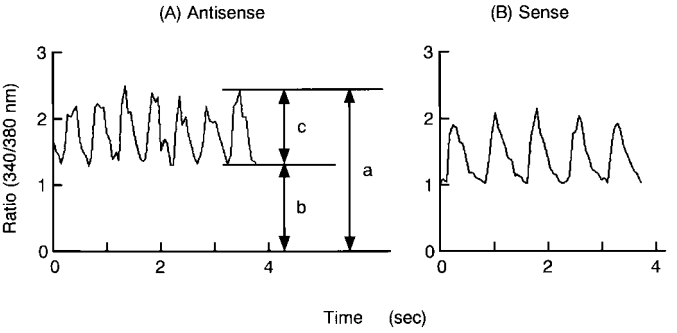

FIG. 1. Effect of antisense oligonucleotides to the cardiac Na^+/Ca^{2+} exchanger on intracellular calcium dynamics during the cardiac cycle. Cardiac myocytes were treated with AS oligonucleotides (A) or S oligonucleotides (B) for 24 h. Data are expressed as fluorescence ratio of 340/380 nm. In (A), "a" or Max refers to the peak systolic value. "b" or Min represents the diastolic value and "c" or $[Ca^{2+}]_i$ transients (Ca-T) is the difference between "a" and "b." All data are expressed as Max, Min and Ca-T and represent the mean of 10 beats.

beating averaged 136 beats/min. Myocytes were treated during 0–24 h with 10 μM AS oligonucleotides, and they were scraped up at various time points. The beating rate of myocytes within 3 h of AS application was 129 ± 13 beats/min and comparable to that at 0 h. For 6 and 12 h of AS oligonucleotides exposure; however, the beating rate increased from 136 ± 7 beats/min to 240 ± 7 beats/min and 234 ± 24 beats/min, respectively. These values represent increase of 76% and 72% in the beating rates at 6 and 12 h, respectively, and are quite consistent with our previous observation that a 24 h exposure to 15 μM concentration of the AS oligo nucleotide led to a mean increase in beating rate of 73% (10). Since the beating rate of the myocytes was so substantially increased even though the reduction in the exchanger protein was less than 20%, it was of interest to assess the effects of the AS treatment on the dynamics of Ca^{2+} regulation in the treated myocytes. Figure 1 shows typical traces of the changes in $[Ca^{2+}]_i$ with the cardiac cycle in AS or S-treated myocytes.

Effects of the Exchanger AS Treatment in $[Ca^{2+}]_i$ Dynamics

Data from $[Ca^{2+}]_i$ dynamics were obtained using digital imaging of changes in $[Ca^{2+}]_i$ through the use of fura-2/AM. After 24 h of exposure, AS oligonucleotides increased the spontaneous beating rate of cardiac myocytes from an average rate of 77 beats to 103 beats per minute (at 37°C), compared to S-treated or untreated controls and confirms our previous findings (10). Data from several experiments are summarized in Table 2. The diastolic $[Ca^{2+}]_i$ is significantly higher in the AS-treated cells, but the magnitude of the Ca^{2+} transient was not markedly affected.

The Possible Site of Action for Na^+/Ca^{2+} Exchanger AS Oligonucleotides

The AS treatment led to a ~20% decrease in expression of Na^+/Ca^{2+} exchanger proteins as assessed by ELISAs within 6–24 h following addition of the AS oligonucleotide (Table 1). However, when the level of message for the Na^+/Ca^{2+} exchanger was assessed using total myocyte RNA, a slight but no statistically significant reduction in exchanger mRNA was detected (Fig. 2). Since the reduction in protein levels was only ~20%, the decrease in mRNA may be too low to be detected by Northern blot analysis (Fig. 3).

DISCUSSION

The results of these studies confirmed our earlier observation that exposure of cardiac myocytes to an AS

TABLE 2

Effect of Antisense Oligonucleotides to the Cardiac Na^+/Ca^{2+} Exchanger on Intracellular Calcium Dynamics and Beating of Neonatal Cardiac Myocytes

Condition	Fluorescence ratio (340/380 nm)			Beating rate (beats/min)
	Min	Max	Ca-T	
Control (n = 51)	1.00 ± 0.02	1.99 ± 0.07	0.99 ± 0.07	77 ± 5
Antisense (n = 52)	1.19 ± 0.04*,#	2.05 ± 0.08	0.89 ± 0.05	103 ± 4*,#
Sense (n = 49)	1.00 ± 0.02	1.91 ± 0.07	0.91 ± 0.05	77 ± 3

Note. Each value represents the mean ± SEM (n = 49–52 cells). Asterisks indicate significant differences between AS-treated cells and control and S-treated cells, respectively: *$P < 0.001$ vs sense, #$P < 0.001$ vs control.

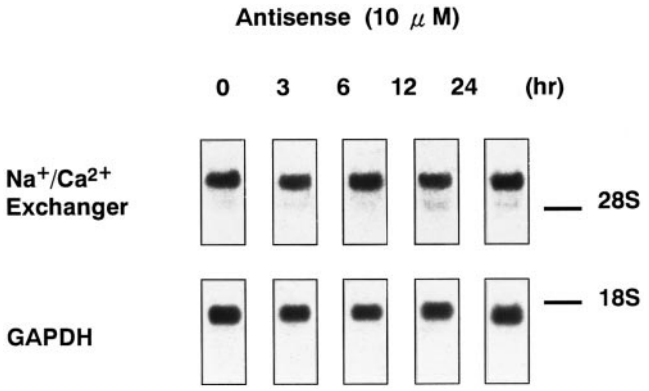

FIG. 2. Time course of Na^+/Ca^{2+} exchanger mRNA expression in antisense-treated myocytes. Cardiac myocytes were treated with AS oligonucleotides (10 μM) for indicated times. Hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe showed equal amount of RNA in each lane. Similar results were obtained from two additional experiments.

oligo that decreases Na^+/Ca^{2+} exchanger activity results in a significant increase in myocytes beating rate (10). In our previous studies the beating rates were determined at 23°C rather than at 37°C as we have done in this study. Thus, even though the overall beating rates were significantly higher at 37°C, the effects of exposure to the AS oligo were virtually identical. The mean increase in beating rate in the AS-treated cells was 72% above controls in the experiments conducted at 23°C, and 74% above controls in assays carried out at 37°C. Following incubation of the myocytes with fura-2, which likely cheletes some of the intracellular Ca^{2+}, the overall beating rate in control cells was decreased from an average of 136 beats/min (Table 1) to 77 beats/min (Table 2). Nevertheless, the cells treated with the AS oligo still exhibited a statistically significant 34% increase in overall beating rate (Table 2). Thus, the observation that reduced expression of the Na^+/Ca^{2+} exchanger leads to an enhanced rate of endogenous beating activity in these immature myocytes is quite reproducible in myocytes irrespective of the reduced free $[Ca^{2+}]_i$ in the presence of the chelating dye fura-2.

Our earlier studies indicated that exposure to the exchanger AS oligo appeared to decrease exchanger activity by ~30%, however, we did not actually determine whether a decrease in either the mRNA or the exchanger protein could be observed. In the present study we examined both protein and mRNA levels in cells exposed to Lipofectamine only, to the S-oligo, and to the AS oligo. As shown in Table 2, the AS treatment led to a time-dependent decrease in the levels of exchanger protein present in the myocytes, with a maximal decrease of ~20% observed 24 h following addition of the AS oligo. However, when Northern blot analysis was used to examine mRNA levels at various times following AS oligo addition to the medium, no significant decrease in the labeling could be detected. Given the relatively small decrease in the levels of exchanger protein in the cells and the relative insensitivity of Northern blot to small changes in mRNA, it is not surprising that no statistically significant reduction in message could be detected. More sensitive techniques will likely be required to detect minor reductions in the mRNA levels. The ~20% decrease in exchanger protein was observed consistently in several different cultures treated with the AS oligo. It is quite

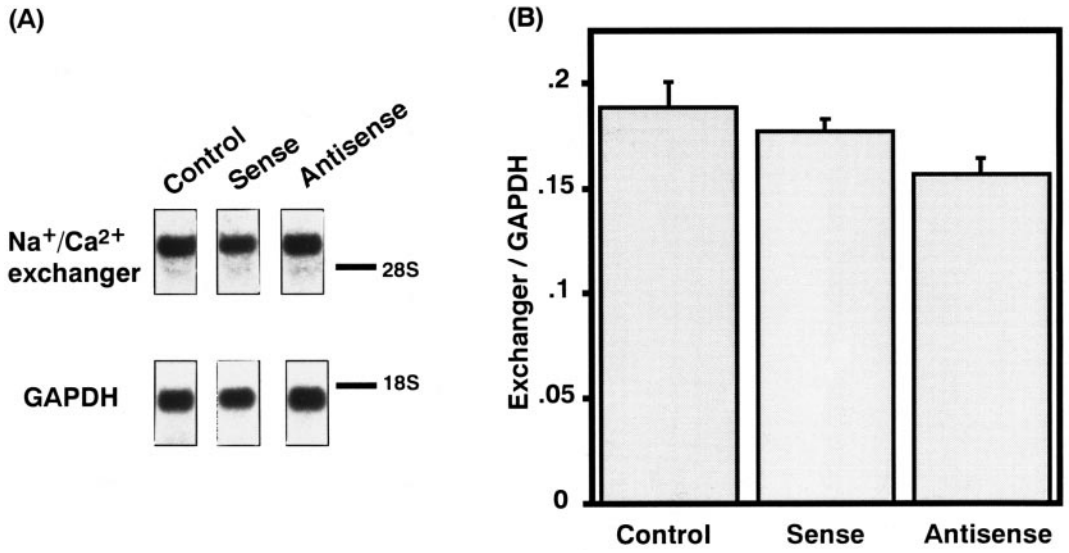

FIG. 3. Northern blot pattern of Na^+/Ca^{2+} exchanger mRNA in cardiac myocytes treated with antisense oligonucleotides. (A) Effect of antisense oligonucleotides to the cardiac Na^+/Ca^{2+} exchanger. Cardiac myocytes were treated with AS oligonucleotides (10 μM), S oligonucleotides (10 μM) or control (Lipofectamine only) for 6 h. (B) Bar graph shows mean values (± SEM) related to GAPDH obtained by densitometric analysis. All data shown were obtained from three different cultures.

surprising that this relatively small reduction in exchanger activity could have such dramatic effects on the myocyte beating rates. These results seem to suggest that this system is major contributor to Ca^{2+} efflux and regulation of the excitability of the immature myocytes. The expression levels of the SR Ca^{2+}-ATPase are quite low in embryonic and early neonatal rabbit myocytes, whereas the Na^{+}/Ca^{2+} exchanger levels are quite high and gradually decrease during development (22–24). Although this may indicate that adult myocytes are less reliant on the exchanger, Lamont and Eisner (25) reported that the exchanger contributes ~ 77% of sarcolemmal Ca^{2+} extrusion following small perturbations in $[Ca^{2+}]_i$. Thus the extent to which our observations regarding the dramatic increase in beating rates in immature myocytes would also apply to mature myocytes has yet to be determined.

A second goal of these studies was to use ratio imaging of free $[Ca^{2+}]_i$ in the beating myocytes to determine the effects of reduced expression of the exchanger on intracellular Ca^{2+} dynamics during the cardiac cycle. AS was shown in the example Fig. 1, the pattern of changes in free $[Ca^{2+}]_i$ during the contraction cycle looks rather uneven and distorted in the AS-treated cells compared to the S-treated myocytes. In addition, the minimal diastolic Ca^{2+} levels reached in AS-treated cells were clearly higher than those in S-treated cells, although the amplitude of the systolic Ca^{2+} peak was not larger in the AS-treated cells.

Changes in diastolic potential play an important role in the control of heart rate (26). The duration of cardiac action potential is determined in part by the preceding diastolic interval, so that when rapid heart rates shorten cycle length, the duration of the action potential is also reduced. The relationship between action potential duration and the length of the preceding diastolic interval arises from several factors. Ca^{2+} plays a critical role in the regulation of ion channel gating in many cell types (27). Increased intracellular Ca^{2+} concentration may accelerate repolarization by opening calcium-activated potassium channels and contribute to early diastolic depolarization, which cause the increasing of heart rate.

These data suggest that the extrusion of Ca^{2+} during diastole is decreased in cells with lower exchanger activity, but the systolic Ca^{2+} signal resulting from Ca^{2+} influx as well as release from the SR is not affected. This observation is consistent with results of other types of studies indicating that the primary role of the exchanger in regulating $[Ca^{2+}]_i$ is to remove Ca^{2+} from the intracellular environment (28–31).

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