Original Paper

Proper Voltage-Dependent Ion Channel Function in Dysferlin-Deficient Cardiomyocytes

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Key Words

Dilated cardiomyopathy • Dysferlin deficiency • Limb-girdle muscular dystrophy type 2B • L-type calcium channel • Mouse model • Ventricular cardiomyocytes • Voltage-dependent ion channels

Abstract:

Background/Aims: Dysferlin plays a decisive role in calcium-dependent membrane repair in myocytes. Mutations in the encoding *DYSF* gene cause a number of myopathies, e.g. limb-girdle muscular dystrophy type 2B (LGMD2B). Besides skeletal muscle degenerative processes, dysferlin deficiency is also associated with cardiac complications. Thus, both LGMD2B patients and dysferlin-deficient mice develop a dilated cardiomyopathy. We and others have recently reported that dystrophin-deficient ventricular cardiomyocytes from mouse models of Duchenne muscular dystrophy show significant abnormalities in voltage-dependent ion channels, which may contribute to the pathophysiology in dystrophic cardiomyopathy. The aim of the present study was to investigate if dysferlin, like dystrophin, is a regulator of cardiac ion channels. **Methods and Results:** By using the whole cell patch-clamp technique, we compared the properties of voltage-dependent calcium and sodium channels, as well as action potentials in ventricular cardiomyocytes isolated from the hearts of normal and dysferlin-deficient (*dysf*) mice. In contrast to dystrophin deficiency, the lack of dysferlin did not impair the ion channel properties and left action potential parameters unaltered. In connection with normal ECGs in *dysf* mice these results suggest that dysferlin deficiency does not perturb cardiac electrophysiology. **Conclusion:** Our study demonstrates that dysferlin does not regulate cardiac voltage-dependent ion channels, and implies that abnormalities in cardiac ion channels are not a universal characteristic of all muscular dystrophy types.

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Introduction

Dysferlin is a member of the ferlin family of proteins. It is a membrane-spanning protein with multiple C2 domains that bind calcium, phospholipids, and proteins to set off signaling events, vesicle trafficking, and membrane fusion [1, 2]. In both skeletal and cardiac muscle cells dysferlin plays a decisive role in calcium-dependent membrane repair [3–5]. Consequently, injuries of the sarcolemma trigger the accumulation of dysferlin-enriched membrane patches and resealing of the membrane [3]. Mutations in the dysferlin gene (*DYSF*) result in the development of a number of progressive muscular dystrophies—the so-called “dysferlinopathies”, an example of which is limb-girdle muscular dystrophy type 2B (LGMD2B) [6]. Besides the relatively well characterized skeletal muscle degenerative processes, it has more recently become apparent that dysferlin deficiency is also associated with cardiac complications. Thus, both in LGMD2B patients [7, 8] and dysferlin-deficient mouse models [4, 7, 9] the development of a dilated cardiomyopathy was observed.

We and others have recently reported that dystrophin-deficient ventricular cardiomyocytes isolated from the hearts of mouse models of Duchenne muscular dystrophy (mdx, only dystrophin-deficient; mdx-utr, dystrophin- and utrophin-deficient) [10–13] show significant abnormalities in voltage-dependent ion channel expression and function. These abnormal “dystrophic” cardiac ion channel properties, i.e. enhanced L-type $\text{Ca}_v1.2$ calcium currents [12, 13], slowed $\text{Ca}_v1.2$ channel inactivation [12, 14, 15], and reduced $\text{Na}_v1.5$ sodium currents [10, 11], may contribute to the pathophysiology in dystrophic cardiomyopathy. Direct regulation of $\text{Ca}_v1.2$ channels by dystrophin accords with the finding that, in mouse cardiomyocytes, $\text{Ca}_v1.2$ colocalizes with dystrophin [14].

In contrast to the established channel regulatory effects of dystrophin, regulation of $\text{Ca}_v1.2$ and/or other cardiac voltage-dependent ion channels by dysferlin has not been studied as yet. Thus, it is unknown if dysferlin-deficiency impairs cardiac ion channel function, a potential pathophysiological mechanism in the dysferlinopathies. The following lines of evidence point to a potential regulatory effect of dysferlin on $\text{Ca}_v1.2$ channels: First, in skeletal muscle, dysferlin colocalizes with the L-type calcium channel [16, 17], and muscle membrane repair requires an interplay between these two proteins [18]. Secondly, the dysferlin interaction partner AHNK [19, 20] associates with regulatory β -subunits of $\text{Ca}_v1.2$ channels [21, 22], and thirdly, the $\alpha_2\delta_1$ calcium channel subunit, which modulates the channel's function [23], is significantly downregulated in the dysferlin-deficient heart (NCBI GEO profile: [http://www.ncbi.nlm.nih.gov/geoprofiles?term=GDS1247\[ACCN\]+cacna2d1,ref.\[24\]](http://www.ncbi.nlm.nih.gov/geoprofiles?term=GDS1247[ACCN]+cacna2d1,ref.[24])). Finally, in the case of dysferlin absence from skeletal muscle cells, mechanical stress triggered abnormally increased calcium influx to the cytosol mediated by the L-type calcium channel, thereby disrupting calcium homeostasis and excitation-contraction coupling [25, 26]. To the best of our knowledge, there is no further evidence for potential regulatory effects of dysferlin on other cardiac ion channels than the L-type calcium channel as yet. In this study, we investigated if dysferlin-deficiency impairs cardiac electrophysiological properties by using a mouse model for LGMD2B.

Materials and Methods

Animal procedures adhered to the guiding principles of the Declaration of Helsinki and met the criteria of our Medical University's Animal Welfare Committee. The Austrian Ministry for Science and Research granted the performed animal experiments (BMWF-66.009/0211-II/10b/2009).

Mouse models

Female wild type (wt) C57BL/10 mice, and dysferlin-deficient C57BL/10.SJL-Dysf mice [27, 28] in an age range between 15 and 20 months were used for the investigation. This advanced animal age was chosen because significant pathological changes are only present in the hearts of aged dysferlin-deficient mice [4]. Throughout the text, the dysferlin-deficient mouse model was termed “*dysf*”. The mice were genotyped by standard PCR-assays.

Isolation of adult ventricular cardiomyocytes

Mice were killed by cervical dislocation, and cardiomyocytes were isolated from the ventricles of their hearts by using a Langendorff setup as previously described [10]. Briefly, hearts were excised by thoracotomy and a cannula was inserted into the aorta to allow for retrograde perfusion with calcium-free solution (in mM: 134 NaCl, 11 glucose, 4 KCl, 1.2 MgSO₄, 1.2 Na₂HPO₄, 10 2, 3-butanedione monoxime, 10 Hepes, LiberaseTH (0.17 mg/ml) (Roche), pH adjusted to 7.35 with NaOH). Hearts were perfused for 18 min at 37°C. The ventricles were cut in pieces and incubated at 37°C on a shaker to stepwise increase the calcium concentration over one hour (final concentration, 200 μM). Digested tissue pieces were triturated to dissolve away cardiomyocytes. The cell suspension was then centrifuged (3 min, 500 rpm), the Langendorff solution was removed, and cells were resuspended in Minimum Essential Medium alpha (Gibco) with added: ITS media supplement (final compound concentrations: 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml selenite), 4 mM L-glutamine, 50 u/ml penicillin, 50 μg/ml streptomycin, and 25 μM blebbistatin. Cells were plated on dishes coated with Matrigel (Becton Dickinson). The chemicals originated from Sigma if not specified otherwise.

Cellular electrophysiological studies

Recordings of calcium, barium, and sodium currents, and of action potentials were made in the whole-cell configuration of the patch-clamp technique in the voltage- and current-clamp mode, respectively. A detailed description of the electrophysiological methods applied can be found in our previous studies [10,12]. Briefly, measurements were performed at room temperature (22 ± 1.5°C) up to 10 hours after isolation of ventricular cardiomyocytes. Glass pipettes pulled from aluminosilicate glass (AF150-100-10; Science Products) on a P-97 horizontal puller (Sutter Instruments) had series resistances between 0.8 – 1.5 MΩ when filled with the respective internal solutions and immersed into the bath. The compositions of the recording solutions (concentrations in mM) are given in Tables 1 and 2.

Voltage and current signals were recorded with an Axoclamp 200B patch-clamp amplifier (Axon Instruments), low-pass filtered at 1–10 kHz, and digitized at 10–100 kHz with a 12-bit A-D/D-A interface

Table 1. External solutions

	Barium	Calcium	Sodium	Action potential
CaCl ₂	-	10	1	2
BaCl ₂	10	-	-	-
MgCl ₂	-	-	1	2
NaCl	-	-	15	140
NMDG	-	-	125	-
KCl	-	-	2.5	4
TEA-Cl	145	145	-	-
Hepes	10	10	10	5
Glucose	-	-	-	5
pH	7.4 (TEA-OH)	7.4 (TEA-OH)	7.4 (H-Cl)	7.4 (Na-OH)

Table 2. Internal solutions

	Barium/Calcium	Sodium	Action potential
Cs-aspartate	145	-	-
CsF	-	105	-
KCl	-	-	140
NaCl	-	10	10
MgCl ₂	2	-	1
Hepes	10	10	10
Cs-EGTA	0.1	-	-
Mg-ATP	2	-	5
Na-GTP	-	-	0.1
EGTA	-	10	2
pH	7.4 (Cs-OH)	7.3 (Cs-OH)	7.2 (K-OH)

(Digidata 1200; Axon Instruments). Data acquisition and analysis was performed with pClamp 6.0 and 10.2 (Axon Instruments) and GraphPad Prism 5.01 (San Diego, USA) software.

Current-voltage (IV) relationships were fit using $I = \sum_{i=1, n} G_{\max, i} * (V - V_{\text{rev}}) * (1 - 1 / (1 + \exp((V - V_{0.5, i}) / K_i)))$, with n equaling 1 and 2 for barium and sodium, as well as calcium currents, respectively. $G_{\max, i}$, maximum conductance(s); V , membrane potential; V_{rev} , reversal potential; $V_{0.5, i}$, voltage(s) of half-maximum activation; K_i , slope factor(s). Barium current inactivation kinetics (representing voltage-dependent calcium channel inactivation) was analyzed by fitting the current decay after channel activation with a single-exponential function to derive time constants (τ -values). Calcium current inactivation kinetics (representing both calcium- and voltage-dependent calcium channel inactivation) was analyzed by measuring the time span between the peak current, and the time point at which the current had decayed to 50%. This parameter is termed "decay half-time".

Action potentials (APs) were elicited both at 1 and 5 Hz by rectangular 4-ms current pulses at 125% threshold level. The maximum upstroke velocity (max. dV/dt) was determined at the maximum of the voltage signal's time derivative. AP duration was analyzed from the maximum depolarization value reached to the time point at which 25% (APD25) and 75% (APD75) of repolarization had been achieved. AP area was determined by integration from the start of the current stimulus to the end of repolarization and is given in $\text{mV} \cdot \text{ms}$.

Electrocardiogram recordings

Standard 6-lead surface electrocardiograms (ECGs) were recorded from anesthetized adult wt and *dysf* mice. To allow for good data comparability, the same animals from which ECGs had been recorded were later (after a recovery time of 2-3 days) used for the cellular electrophysiological studies. The mice were anesthetized with a single ip injection of chloral hydrate (350 mg/kg). The depth of anesthesia was checked (minimal response to hind-foot pinch). Small-needle ECG leads were placed subcutaneously on all 4 extremities, and, by using a heating pad and lamp, the body temperature was maintained at 37°C. The signals were amplified (Gould model 11 G412301; Gould, Cleveland, OH) and high- and low-pass filtered with -3 dB cut-off frequencies of 0.3 and 1 kHz, respectively. Signals were digitized at 5 kHz, and stored for offline analyses. ECG signals were averaged over approximately 100 beats prior to evaluation in order to reduce noise levels. The ECG interval values were determined by calculation of the mean of all standard leads. Individual values which could not be clearly determined were excluded from analysis. The RR interval was identified as average distance between 100 successive QRS complexes. Heart rate was then calculated as $60,000/\text{RR}$ (ms) expressed in beats per minute (bpm). The ECG intervals (QRS, PQ, and QT) were determined as indicated in Fig. 4A.

Statistical analyses

Data are expressed as means $\pm$ SEM. Statistical comparisons between wt and *dysf* mice were made using an unpaired two-tailed Student's t -test. A p value < 0.05 was considered significantly different *; $p < 0.01$ highly significant difference **.

Results

Calcium channels in wt and *dysf* cardiomyocytes

We and others have recently reported that dystrophin-deficient cardiomyocytes derived from adult mdx and mdx-utr mice (mouse models of Duchenne muscular dystrophy) have significantly enhanced L-type calcium currents [12, 13]. Moreover, calcium channel inactivation was impaired in dystrophic myocytes [12, 14, 15]. Here we tested if cardiomyocytes derived from *dysf* mice exhibited similar abnormalities in currents through calcium channels (Fig. 1, Table 3). It can be observed that, irrespective of the use of barium (Fig. 1A-C) or calcium (Fig. 1D-F) as charge carrier, the current densities and properties were similar in wt and *dysf* cardiomyocytes (Figs. 1B and E, Table 3). In addition, when using calcium as charge carrier, besides L-type calcium currents, also T-type calcium currents (left current peak in Fig. 1E) could be detected. As the L-type currents, also the T-type currents were similar in wt and *dysf* myocytes (Fig. 1E, Table 3). Finally, a comparison of the kinetics

Fig. 1. Calcium channel properties in wild type (wt) and *dysf* cardiomyocytes. A. Original traces of barium currents of a typical wt cardiomyocyte elicited by the pulse protocol given on top. B. Current density-voltage relationships of wt and *dysf* cardiomyocytes (left). On the right, a comparison of the current densities at +20 mV is shown ($p=0.44$). C. Comparison of the barium current decay kinetics between wt and *dysf* cardiomyocytes at various membrane voltages (p always >0.35). τ -values were derived from single-exponential fits of the current decay after channel activation. D. Original traces of calcium currents of a typical wt cardiomyocyte elicited by the pulse protocol displayed on top. E. Current density-voltage relationships (left) and respective current densities for T-type (at -20 mV) and L-type (at +30 mV) calcium channels (right) in wt ($n=11$) and *dysf* (13) cardiomyocytes. F. Calcium current decay kinetics (decay half-times) in wt and *dysf* cardiomyocytes at various voltages ($p>0.16$). Fitting the data points of the current-voltage relationships in B and E revealed the parameters given in Table 3.

Table 3. Calcium channel properties. $V_{0.5'}$ voltage of half-maximum activation; K, slope factor; $V_{rev'}$ reversal potential; n, number of experiments performed. T- and L-Type calcium current data points (Fig. 1E) were fit separately (for equation see Materials and Methods). No significant difference in any of the current parameters existed between wt and *dysf* cardiomyocytes (p -values >0.05).

		$V_{0.5}$ (mV)	K (mV)	V_{rev} (mV)	n
Barium currents					
wt	L-type	0.8 ± 0.9	5.7 ± 0.6	61 ± 1.5	15
<i>dysf</i>	L-type	3.8 ± 0.8	5.5 ± 0.5	62 ± 1.3	11
Calcium currents					
wt	T-type	-24 ± 2.4	5.5 ± 0.9	-	11
	L-type	16 ± 1.8	8.4 ± 1.3	83 ± 2.6	11
<i>dysf</i>	T-type	-24 ± 3.7	5.5 ± 1.4	-	13
	L-type	16 ± 2.2	7.3 ± 1.5	86 ± 4.3	13

of the current decay after L-type calcium channel activation in wt and *dysf* cardiomyocytes revealed no significant differences. This was true for barium (Fig. 1C) and calcium (Fig. 1F) currents, suggesting that both voltage-dependent and calcium-dependent channel inactivation were similar in wt and *dysf* cardiomyocytes. Together these data suggested that *dysf* cardiomyocytes do not show calcium channel abnormalities.

Fig. 2. Sodium channel properties in wt and *dysf* cardiomyocytes. A. Original current traces of a typical wt cardiomyocyte elicited by the pulse protocol displayed on top. B. Current density-voltage relationships derived from a series of experiments ($n=14$ for wt and 16 for *dysf*) as shown in A. Fitting of the data points revealed the following parameters (in mV) for wt ($V_{0.5} = -42 \pm 0.5$, $K = 4.8 \pm 0.4$, $V_{rev} = 25 \pm 0.9$) and *dysf* ($V_{0.5} = -36 \pm 0.6$, $K = 5.2 \pm 0.4$, $V_{rev} = 25 \pm 0.9$) cardiomyocytes. The only significant difference ($p < 0.01$) existed between the $V_{0.5}$ values of wt and *dysf* cells. C. Analysis of the current densities at -25 mV in wt and *dysf* cardiomyocytes. There was no significant difference ($p = 0.32$).

Fig. 3. Action potentials (APs) in wt and *dysf* cardiomyocytes. A. Typical AP recorded from a wt cardiomyocyte at 1 Hz pacing frequency. B. Comparison of maximum AP upstroke velocity (max dV/dt ; left) and AP peak amplitude (right) in wt ($n=18$) and *dysf* (18) cardiomyocytes. No significant difference existed between wt and *dysf* (see respective p values in the graphs). C. Comparison of AP area (left) and AP duration at 25 and 75% repolarization (right) in wt and *dysf* cardiomyocytes. D – F. Comparison of the respective AP parameters (see A – C) in wt ($n=14$) and *dysf* (14) cardiomyocytes at 5 Hz pacing frequency.

Sodium channels in wt and *dysf* cardiomyocytes

Dystrophin-deficient cardiomyocytes derived from mdx and mdx-utr mice showed significantly reduced sodium current densities [10, 11, 29]. Here we tested if the sodium currents were also reduced in cardiomyocytes from *dysf* mice. On the contrary, we found roughly similar current density-voltage relationships in wt and *dysf* cardiomyocytes (Fig. 2B.), whereby a minor $V_{0.5}$ shift toward more positive voltages in *dysf* cells showed up. The current densities analyzed at -25 mV (near the current maxima) were similar in wt and *dysf* cells (Fig. 2C).

Action potentials in wt and *dysf* cardiomyocytes

The cardiomyocyte's action potential (AP) reflects the concerted action of a plethora of different types of ion channels. To detect any potential differences in ion channel

Fig. 4. Surface ECG recordings on wt and *dysf* mice. A. Typical original ECG signals from first standard lead of a wt mouse (right), and the corresponding averaged signal of 100 beats (left). B. Comparison of the QRS (left), PQ (middle), and QT (right) intervals between wt (n=3) and *dysf* (4) mice. The respective intervals were determined as indicated in A. No significant difference existed between wt and *dysf* in any of the intervals detected (see respective p values in the graphs). The heart rates of wt and *dysf* mice were similar (p=0.82), and amounted to 467 ± 28 and 455 ± 36 bpm, respectively.

properties between wt and *dysf* cardiomyocytes, we therefore also studied and compared their APs. Significant AP abnormalities were previously reported for dystrophin-deficient cardiomyocytes [10, 11]. Fig. 3 shows that the APs recorded from wt and *dysf* myocytes at two different frequencies (1 and 5 Hz) were similar. Thus, there was no significant difference between wt and *dysf* in any of the AP parameters analyzed. Parameters mainly reflecting sodium channel activity, i.e. AP upstroke velocity and peak AP amplitude (Figs. 3B and E), were as well similar as AP duration (Figs. 3C and F), which is essentially determined by the activity of L-type calcium and various potassium channels. Since calcium channel properties were normal in *dysf* cardiomyocytes (see above), our AP recordings indirectly suggested that the same is true for the ensemble of repolarizing potassium channels.

Electrocardiograms in wt and *dysf* mice

Similar ion channel and AP properties at the cardiomyocyte level accord with similar electrocardiogram (ECG) parameters in wt and *dysf* mice. In Fig. 4B, the QRS, the PQ, and the QT intervals of the ECG are compared between wt and *dysf* animals. It can be observed that all these parameters were normal in *dysf* mice. A normal QRS interval in *dysf* mice, reflecting the depolarization of the ventricles of the heart, corresponds to the normal AP upstroke velocity and peak AP amplitude observed in *dysf* cardiomyocytes (Figs. 3B and E). The ECG parameters likely to be affected by calcium channels are the PQ and the QT interval. These parameters reflect AV nodal conduction, and ventricular de- and repolarization, respectively. Our finding of similar PQ and QT intervals in wt and *dysf* animals thus accords with normal calcium channel properties in *dysf* cardiomyocytes (Fig. 1, Table 3). Taken together, our ECG recordings suggest normal electrical conduction properties in the heart of *dysf* mice.

Discussion

Dysferlin is strongly expressed in cardiomyocytes [30] and plays a decisive role in calcium-dependent membrane repair [4]. Consequently, it does not surprise that dysferlin-deficiency is linked to cardiomyopathy development in mice [4, 7, 9] and in LGMD2B patients [7, 8]. Whereas dilated cardiomyopathy in the context of dystrophin-deficiency was associated with substantial cardiac voltage-dependent ion channel abnormalities [10–13], here we report basically normal ion channel properties in dysferlin-deficient (*dysf*) cardiomyocytes. Together with normal ECGs in *dysf* mice these results suggest that dysferlin-deficiency does not impair cardiac electrophysiology. Hence two important conclusions

can be drawn: 1) dysferlin, unlike dystrophin, does not regulate cardiac ion channels, and 2) significant abnormalities in cardiac voltage-dependent ion channels do not represent a universal characteristic of all types of muscular dystrophy.

A reasonable explanation for the lack of ion channel abnormalities in dysferlin- as opposed to dystrophin-deficient cardiomyocytes observed in the present study is yet to be found. A conspicuous difference between dysferlin- [4, 9, 31] and dystrophin-deficient [32–36] dystrophic mice is that the latter develop a more severe dilated cardiomyopathy. It is therefore tempting to speculate that the incidence of considerable cardiac electrophysiological abnormalities in a dystrophy mouse model is associated with the severity of the developing dilated cardiomyopathy. An argument supporting this hypothesis can be derived from our earlier work (Koenig et al. 2011 [10]), as well as from Albessa et al. 2011 [11]. Thus, in both studies, more severe sodium channel impairments were found in mdx-utr compared to mdx cardiomyocytes. The mdx-utr mouse model is known to develop a more pronounced dilated cardiomyopathy than mdx mice [33, 35, 36].

A surprising finding of the present study was that dysferlin, unlike dystrophin, does not regulate cardiac L-type calcium channels. We had expected a regulatory effect because the dysferlin interaction partner AHNAK [19, 20] associates with regulatory β -subunits of $\text{Ca}_v1.2$ channels [21, 22], and the $\alpha_2\delta_1$ calcium channel subunit, which modulates the channel's function [23], is significantly downregulated in the dysferlin-deficient heart [24]. In this regard it is worthwhile to consider recent work dealing with dysferlin regulation of calcium signaling and homeostasis in skeletal muscle cells [25, 26]. It was suggested that dysferlin is a t-tubule protein that stabilizes stress-induced calcium signaling. According to that, mechanical stress results in influx of calcium to the cytosol mediated by the L-type calcium channel (adult skeletal muscle isoform, $\text{Ca}_v1.1$) [25, 26]. This calcium influx does not cause significant muscle injury in wt muscle cells. However, when dysferlin is absent, calcium influx is greatly enhanced, disrupting calcium homeostasis and excitation-contraction coupling. Finally, this activates a cascade of calcium-mediated events promoting further damage to the muscle cell including proteolysis and oxidative stress [26]. A significant contribution of L-type calcium channels as pathway for deleterious calcium entry in dysferlin-deficient skeletal muscle cells was proven by showing that the L-type channel blocker diltiazem mitigated damage mediated by mechanical stress [25]. To our knowledge, it is currently unknown if mechanical stress also triggers abnormal calcium entry via L-type calcium channels in dysferlin-deficient cardiomyocytes. If so, it is conceivable that calcium channel function under conditions without mechanical stress is normal in dysferlin-deficient cells. This is in accordance with the findings of the present study. Under mechanical stress, however, L-type calcium channel activity in dysferlin-deficient cardiomyocytes may be abnormally enhanced as in dysferlin-deficient skeletal muscle cells [25, 26]. This could represent a mechanism to explain why dysferlin-deficient mice develop more pronounced cardiac abnormalities when their hearts are challenged by mechanical stress [4, 7].

Acknowledgements

We thank R.E. Bittner for providing the C57BL/10 and the dysferlin-deficient C57BL/10. SJL-Dysf mice. J. Uhrinova and B. Schmolmueller is thanked for technical assistance. The work was funded by the Austrian Science Fund FWF (P23060 to K.H. and W1232-B11 to H.T.).

Disclosure Statement

No conflict of interest.

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