Journal of Physiology (1993), **469**, *pp.* 11–19
With 3 figures
Printed in Great Britain

MEMBRANE POTENTIAL, RESTING CALCIUM AND CALCIUM TRANSIENTS IN ISOLATED MUSCLE FIBRES FROM NORMAL AND DYSTROPHIC MICE

BY STEWART I. HEAD*

From the Department of Zoology, La Trobe University, Bundoora, Melbourne, Victoria 3083, Australia

(*Received 12 August 1992*)

SUMMARY

1. Single skeletal muscle fibres were enzymatically isolated from the flexor digitorum brevis muscles (FDB) of dystrophic *mdx* and control C57BL/10 mice aged 3–9 weeks. In this age range the majority (> 95%) of the *mdx* fibres were morphologically normal.

2. There was no significant difference between the resting membrane potential (RMP) of *mdx* and control mice, $-71{\cdot}2 \pm 1{\cdot}21$ ($n = 26$) and $-70{\cdot}6 \pm 1{\cdot}15$ mV ($n = 42$), respectively.

3. At RMP more negative than -60 mV the resting calcium (recorded with fura-2, free acid ionophoresed into cell) in the dystrophic *mdx* cells was not significantly different from the normal animals, $45{\cdot}7 \pm 4{\cdot}1$ ($n = 10$) and $46{\cdot}2 \pm 3{\cdot}9$ nM ($n = 9$), respectively.

4. The resting cytosolic calcium concentration was measured simultaneously with the RMP. At RMP between -60 to -17 mV there was an increase in the resting calcium concentration in both *mdx* and control ranging from 79·3 to 252 nM. This increase was most probably due to the activation of the slow calcium current.

5. Fura-2 calcium transients were produced via single action potential stimulation using an intracellular microelectrode both to stimulate the cell and record potential changes. There was no significant difference between the rise time (T_p) or half-decay time ($T_{\frac{1}{2}}$) at 22 °C of the calcium transient in response to a single action potential in *mdx* compared to normal animals, $5{\cdot}9 \pm 0{\cdot}34$ ($n = 8$) and $5{\cdot}4 \pm 0{\cdot}36$ ms ($n = 7$); $39{\cdot}5 \pm 2{\cdot}9$ ($n = 8$) and $40{\cdot}75 \pm 3{\cdot}7$ ms ($n = 7$), respectively.

6. In conclusion it appears that over the age range 3–9 weeks the absence of dystrophin does not affect the RMP, resting calcium or calcium transient parameters of morphologically normal FDB *mdx* fibres.

INTRODUCTION

C57BL/10 *mdx* (*mdx*) dystrophic mice lack the same protein, dystrophin, from their sarcolemma as humans with Duchenne muscular dystrophy (DMD) (Hoffman, Brown & Kunkel, 1987). Due to its similarities to structural proteins such as spectrin

* Present address: School of Physiology and Pharmacology, University of New South Wales, Kensington, PO Box 1, NSW 2033, Australia.

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it has been hypothesized that dystrophin plays a role in mechanically stabilizing the muscle membrane (Brown & Hoffman, 1988). Dystrophin-negative myotubes and adult muscle fibres have been shown to have an elevated resting cytosolic [Ca^{2+}] (Mongini *et al.* 1988; Turner, Westwood, Regen & Steinhardt, 1988; Fong, Turner, Denetclaw & Steinhardt, 1990). This elevated calcium concentration could arise either because the dystrophin-deficient membranes are more fragile allowing calcium entry (Menke & Jockusch 1991), or because of abnormal functioning of calcium ion channels in the sarcolemma. Functionally abnormal calcium channels have been found in myotubes grown from *mdx* mice and DMD humans (Fong *et al.* 1990; Franco & Lansman, 1990) and result in calcium entry when the myotubes are at rest. However, these channels have not been found in dystrophin deficient adult *mdx* muscle cells (Haws & Lansman, 1991). In the adult *mdx* mice between 2–5 weeks of age the proximal limb muscles undergo a process of degeneration which affects up to 100% of the fibres in some muscles (Carnwath & Shotton, 1987). The majority of the repaired or regenerated fibres are morphologically deformed (Head, Williams & Stephenson 1992) and appear to persist for the normal lifespan of the animal (DiMario, Uzman & Strohman, 1991).

In the present study I demonstrate that the morphologically normal skeletal muscle fibres present in the FDB from *mdx* animals aged 3–9 weeks of age have a normal RMP and do not exhibit elevated levels of free cytoplasmic calcium. In addition the calcium transient parameters in the dystrophin-negative fibres are the same as those in the control dystrophin-positive cells suggesting that the absence of dystrophin which may also be associated with the transverse tubular system (Knudson, Monaco, Kahl & Campbell, 1988) does not effect the excitation–contraction coupling process.

METHODS

Muscle fibre isolation

Control (C57BL/10ScSn) and *mdx* (C57BL/10 *mdx*) mice aged 3–9 weeks were killed by cervical dislocation. The FDB (predominantly fast-twitch) muscle was removed and incubated for 1 h at 35 °C in Tyrode solution (mM): 2·5 Ca^{2+}, 10 glucose, 135 NaCl, 4 KCl, 1 $MgCl_2$, 0·33 NaH_2PO_3, 10 Hepes (*N*-2-hydroxyethylpiprazine-N′-2-ethanesulphonic acid), (pH 7·3) with 0·2% collagenese IV (Sigma Chemical Co., St Louis, MO, USA). The muscle mass was then removed from the solution containing collagenase and washed twice in Tyrode solution. At all times the muscles were exposed to 2·5 mM Ca^{2+}. Single fibres were obtained by gently pipetting the muscle mass (Head, Stephenson & Williams, 1990).

Electrophysiology

The RMP of the fibres was measured on an Axoclamp 2A (Axon Instruments, USA) using intracellular glass microelectrodes filled with 3 M KCl (23–30 MΩ). The intracellular electrode was used to stimulate the muscle fibres with a brief (1 ms) depolarizing pulse. The ionized form of the calcium-sensitive dye fura-2 (Molecular Probes, OR, USA) was ionophoresed into the muscle fibres to give a final concentration of 5–50 μM fura-2 in the cell (Williams, Head, Bakker & Stephenson, 1990). Fura-2 (1 mM) in DH_2O was introduced into the tip of the ionophoretic electrode and the shank was then filled with 150 mM potassium acetate. After filling with fura-2 the fibres were left for 20 min before any readings were taken to allow for complete distribution of the dye in the myoplasm.

Fluorescence measurements

The individual muscle fibres were viewed with a Nikon 20 UV-F objective on a Leitz inverted microscope equipped for xenon epifluorescence. At this magnification a single muscle cell (about 0·3 mm) fitted into the field of view. The fura-2 in the muscle cells was excited at 350 and 380 nm.

Before each recording from a fibre the auto-fluorescence was measured and subtracted from the actual fluorescence recording. The fluorescent signal was filtered through a long-pass 510 nm filter and its intensity was measured with a photomultiplier tube. The photomultiplier tube current was digitized and stored on a IBM compatible 386 computer for later analysis. The ratios of the fluorescent signals following excitation at 380 and 350 nm were recorded. Calcium concentrations were calculated from this ratio with the following equation (Grynkiewicz, Poenie & Tsien, 1985).

$$[Ca^{2+}] = K_d \beta (R - R_{min})/(R_{max} - R), \quad (1)$$

where R is the 350:380 nm emission ratio measured in the cell. R_{max} is the maximum ratio at saturating $[Ca^{2+}]$ and R_{min} is the minimum ratio under calcium free conditions. K_d is the apparent dissociation constant of fura-2 and β is the ratio of the 380 nm fluorescence under minimum and maximum $[Ca^{2+}]$ conditions. $K_d\beta$ was measured in extensor digitorum longus muscle fibres (Bakker, Head, Williams & Stephenson 1992). R_{max} and R_{min} were measured in fura-2-loaded FDB fibres treated with the ionophore Br-A23187 under conditions of saturating (pCa 4·5) ($n = 4$, 2 control, 2 *mdx*) and zero calcium (pCa > 9) ($n = 4$, 2 control, 2 *mdx*). Calcium transients were measured in the FDB muscle fibres by recording the fluorescent intensity changes to two successive depolarizing 1 ms pulses (producing a single action potential in each case) at 380 and 350 nm. The digitized fluorescent readings were then read into a spreadsheet program, the 350 and 380 nm transients were digitized and the above equation was used to produce the actual calcium transient.

Statistics

Data are presented as means ± standard error of the mean (S.E.M.). Student's t test was used to determine the statistical significance at a significance level of 0·05.

RESULTS

RMP and cytosolic calcium

Of 580 *mdx* FDB skeletal muscle fibres viewed in this study only four had deformities; this is in agreement with our previous study where in mice younger than 14 weeks of age less than 5 % of the *mdx* FDB fibres were deformed (Head *et al.* 1992). Only fibres of a normal appearance were used in the present study. Of the 525 fibres viewed from the FDB of normal animals none was observed to have any deformities. There was no significant difference ($P > 0·2$) between the RMP recorded in muscle fibres from normal animals compared with the RMP recorded from the dystrophic *mdx* myotubes (Table 1). The RMP and resting cytosolic calcium were measured simultaneously in both dystrophic and normal cells. At resting potentials more depolarized than −60 mV there was a positive correlation ($r = 0·44$) between the RMP of the cell and its resting calcium concentration (Fig. 1). The resting calcium values in these cells ranged from 79·3 to 252 nM. However, these concentrations were not large enough to cause the cells to contract (Head *et al.* 1990) and visually the depolarized (> −60 mV) cells were indistinguishable from the more hyperpolarized cells (< −60 mV). This elevated intracellular calcium is most probably due to the slow calcium current activated by depolarization of these cells (Lamb & Walsh, 1987). When cells with a more negative RMP are considered, potentials at which the calcium current would not be activated, then the resting cytosolic calcium does not alter with the RMP (Fig. 1).

Resting $[Ca^{2+}]$

In considering the actual value of the resting calcium and time course of the $[Ca^{2+}]$ transient in the FDB muscles from dystrophic and normal animals all fibres with a RMP more positive than −60 mV were not considered. The values R_{min} and R_{max} (see eqn (1), Methods) were measured in the *mdx* and normal FDB fibres. There was

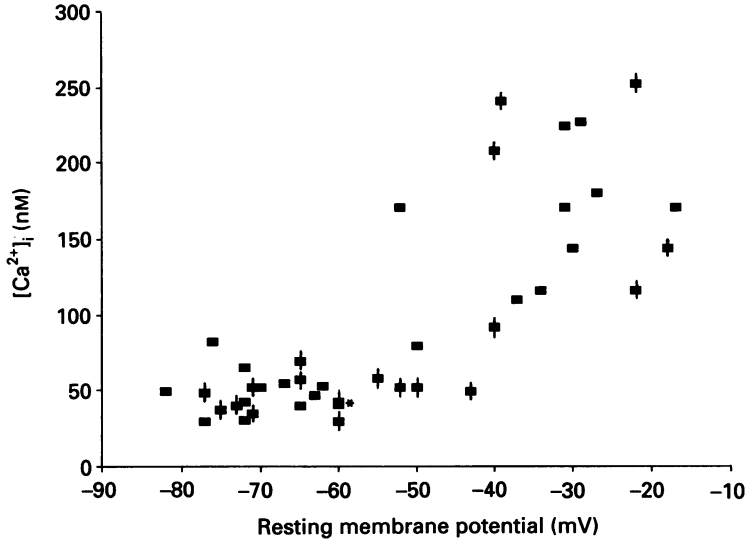

Fig. 1. The resting calcium and RMP were monitored simultaneously in 22 (■) control and 19 (▮) *mdx* cells. Note that at potentials more positive than −60 mV there was an increase in the resting calcium in these more depolarised cells. *Two points overlayed.

TABLE 1. The RMP, $[Ca^{2+}]_i$ and the time to peak (T_p) and half-decay time ($T_{\frac{1}{2}}$) of the calcium transient

	RMP (mV)	$[Ca^{2+}]_i$ (nM)	T_p (ms)	$T_{\frac{1}{2}}$ (ms)
Control	−70·6 ± 1·15	46·2 ± 3·9	5·4 ± 0·36	40·75 ± 3·7
n	26	9	7	7
mdx	−71·2 ± 1·21	45·7 ± 4·1	5·9 ± 0·34	39·5 ± 2·9
n	42	10	8	8

There was no significant difference ($P < 0·2$) between any of these parameters in *mdx* compared to normal mice.

no significant difference between these parameters in normal and *mdx* fibres. If an accurate quantitative measure of the resting $[Ca^{2+}]$ is to be made, these parameters have to be measured in the cell type being used (Bakker *et al.* 1992). The ionophoresis procedure used in this study allowed the concentration of fura-2 internalized in the cytoplasm of the cell to be controlled. Fura-2 concentrations were maintained at values below 50 μM, a concentration which would not alter the resting $[Ca^{2+}]$ (Williams *et al.* 1990). This assumption was confirmed by reinserting the ionophoresis microelectrode after an initial resting $[Ca^{2+}]$ value was obtained and increasing the cytoplasmic values of fura-2 to ≈ 200 μM. There was no difference in the resting calcium after this procedure ($[Ca^{2+}]$ 46 ± 1·5 nM, $n = 4$). There was no significant difference between the resting calcium in the FDB fibres from the normal compared with the *mdx* animals (Table 1).

Calcium transients

Calcium transients were recorded from cells which had a cytoplasmic fura-2 concentration of less than 50 μM. This concentration of fura-2 should not significantly

buffer the parameters of the transients measured (Noble & Powell, 1991). The muscle fibres were firmly attached to the glass coverslip so that the twitch occurred under almost isometric conditions. Any artifacts generated by movement of the fibres were minimal as indicated by the small change in the 350 nm emission intensity (which is

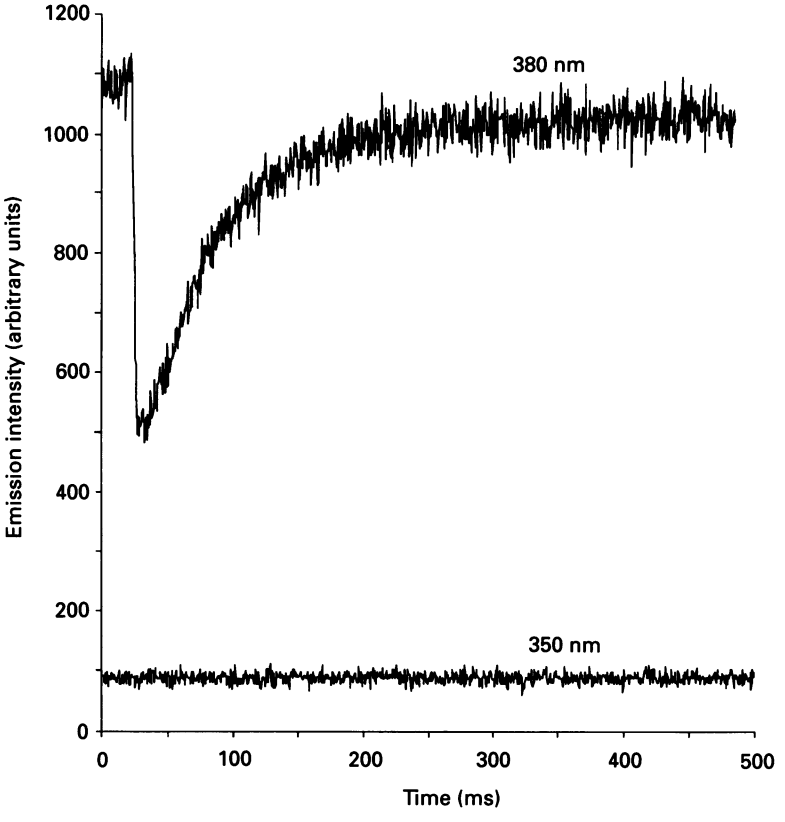

Fig. 2. The change in 510 nm emission intensity when a FDB *mdx* muscle is stimulated at excitation wavelengths of 380 and 350 nm. The trace at each wavelength is a response to a 1 ms depolarizing stimulus which produced a single action potential in each case. Note the lack of movement artifact associate with the 350 nm trace. The cell had a RMP of −73 mV which remained constant for the two stimulations showing that the cell had not been damaged by movement about the intracellular recording/stimulation electrode. In each case the cell was seen to twitch strongly. Due to the strong attachment of the fibre to the glass coverslip the twitch was close to isometric.

close to the isosbestic point for fura-2) during the twitch. The peak calcium during this twitch was 745 nM and the cell was visually seen to twitch in each case (350/380 nm, Fig. 2). The fibres were stimulated with an intracellular microelectrode in bridge mode which was also used to measure simultaneously the membrane potential of the fibre. All fibres used in the present analysis had membrane potentials which returned to resting levels after the twitch. Each fibre was stimulated with no more than eight depolarizing pulses. Fibres were viewed during stimulation to see whether or not they were twitching. Only cells with a twitch calcium peak greater

than 350 nM, such as those illustrated in Fig. 3, were observed to move. The calcium transients measured in this study were variable in amplitude 280–770 nM (Fig. 3). This variability was the same in fibres from control animals and in *mdx* muscle fibres. The time to peak and half-decay time of the twitch calcium transients measured in

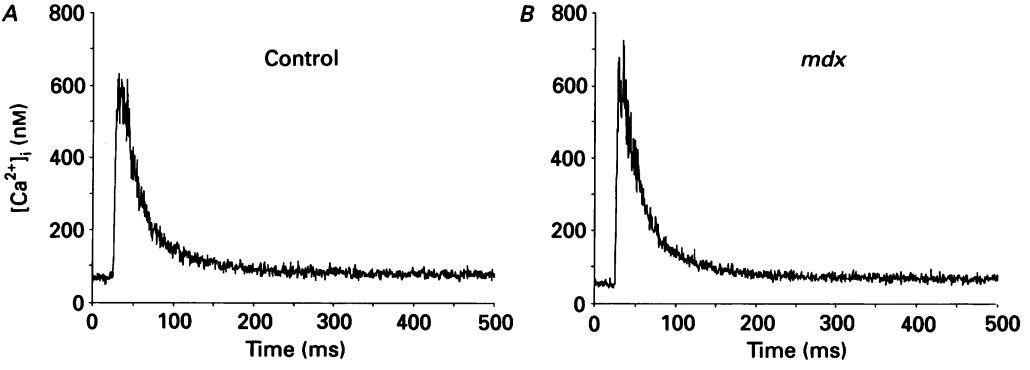

Fig. 3. Two representative calcium transients from a control (*A*) and *mdx* (*B*) FDB skeletal muscle fibre. There was some variation in the transient peak, but the rise time and half-decay time were not significantly different in each case. The RMP of the cell in *A* was −72 mV and that in *B* was −69 mV. Both cells were firmly attached to the glass coverslip and were observed to twitch.

the *mdx* cells was not significantly different from the time to peak and half-decay time measured in muscle cells from control animals (Table 1). It must be noted that the off rate constant k_1 for the fura-2–Ca^{2+} reaction has been measured in the cytoplasm of frog skeletal muscle fibres as 23 s^{-1} (Baylor & Hollingworth, 1988). This means that if a similar figure is assumed for mammalian myoplasm then values for $T_{\frac{1}{2}}^{i}$ measured in the present study are approaching their effective limits, and it follows that it would be hard to detect decreases in the rate of decay of the transients.

DISCUSSION

RMP

The average RMP recorded from the FDB muscle of −71·2 for *mdx* and −70·6 mV for control correlates well with published values of −73 and −75 mV from non-digested normal and *mdx* extensor digitorum longus muscle (EDL) reported by Hollingworth, Marshall & Robson (1990) and values of −78 and −75 mV reported for *mdx* and normal epitrochleoanconeus muscle by Lyons & Slater (1991). The RMP recorded in the present study is slightly more negative than the RMP recorded in dystrophin-positive rat FDB fibres produced by the digest method (Bekkof & Betz, 1977), which suggests that the enzyme digest technique used in the present study does not adversely affect the RMP in either normal or dystrophic *mdx* muscle cells. All the above readings are, however, more depolarized than readings from non-digested control (−98 mV) and *mdx* (−95 mV) EDL fibres reported by Mathes, Bezanilla & Weiss (1991). The more hyperpolarized values in this study may be the result of the lower chloride concentrations in the saline

solution. The results from the present investigation is supported by the findings of all the above studies in clearly showing that the RMP of dystrophin-negative skeletal muscle fibres is the same as that of fibres from normal animals.

Resting intracellular calcium

The values reported for resting levels of ionized calcium in the cytoplasm of skeletal muscle fibres using the calcium-sensitive dye fura-2 have ranged from negative values(!) (Suda & Kurihara, 1991) to 120 nm (Turner, Fong, Denetclaw & Steinhardt 1991) with a range of intermediary values (e.g. Williams *et al.* 1990; Westerblad & Allen, 1991). These variations may arise due to a variety of reasons. There are, however, two main sources of error which should be considered. (a) Use of the membrane-permeable form of fura-2 (e.g. fura-2 AM). Unless care is taken with the loading protocol (Williams & Fay, 1990) the dye can load into intracellular stores of calcium such as the sarcoplasmic reticulum. In addition, if the dye is not completely cleaved in the cytoplasm the uncleaved dye will give a fluorescent signal which is not related to the concentration of calcium ions in the cell. (b) By applying published values for the constants used to convert the ratio values into $[Ca^{2+}]$ (eqn (1)). In this study these problems are largely eliminated by measuring the constants R_{max} and R_{min} (eqn (1)) in the muscle cell and by using the membrane-impermeable form of fura-2. The average resting calcium concentration reported for the morphologically normal muscle obtained from this age group of mice was 46·2 nM which was not significantly different from the average resting calcium concentration found for the *mdx* skeletal muscle cells of 45·7 nM. Turner *et al.* (1988) in a study measuring the calcium in FDB murine muscle showed that *mdx* FDB fibres had an elevated $[Ca^{2+}]_i$ of 90 nM (cf. 40 nM in controls). The difference between their findings and those of the present study probably arises because their recordings were made from fibres in the intact muscle and therefore it is not possible to differentiate morphologically normal fibres from deformed ones. In addition, they did not simultaneously measure the RMP so it is possible, as discussed below, that their *mdx* values were elevated due to the inclusion of a significant proportion of depolarized fibres as is suggested by the larger standard deviation (S.D.) seen in their *mdx* recordings ($\pm$46 nM) compared to control S.D. ($\pm$14 nM). The question of whether or not the absence of dystrophin in adult *mdx* muscle results in an increased calcium influx at rest remains open: Turner *et al.* (1991) using calcium imaging techniques and the calcium-sensitive dye fura-2 claim that there is a higher calcium flux at the sarcolemma in *mdx* mice compared with control. However, care must be taken in interpreting these results due to artifacts associated with edge effects in fura-2 measurements (Williams *et al.* 1990). In addition McArdle, Edwards & Jackson, (1992) in a study using radioactive $^{45}Ca^{2+}$ showed that the rate of calcium influx was the same in *mdx* muscle as in control.

Resting intracellular calcium versus RMP

One of the most interesting findings of the present study was the result that at RMP more depolarized than -60 mV resting intracellular calcium was elevated both in control and *mdx* fibres. This elevated calcium is probably the consequence of the activation of the slow calcium current at these potentials. This slow calcium current

has been shown to be three times as large in fast-twitch fibres (e.g. FDB muscle) in comparison with slow-twitch muscle fibres (Lamb & Walsh, 1987). This dependence of calcium on RMP at depolarized potentials illustrates how important it is to measure simultaneously the RMP and calcium as even at the highest recorded [Ca^{2+}] (252 nM) the cells were not contracted and had a normal striation pattern.

Calcium transients in normal and mdx *muscle*

There was no difference in the time to peak or half-decay time between normal and *mdx* calcium transients in response to a single twitch. Turner *et al.* (1991) showed that in *mdx* fibres with an elevated resting [Ca^{2+}] the calcium transient in response to stimulation was prolonged, compared with transients measured in fibres from normal control muscles. However, when they reduced the intracellular calcium in the *mdx* fibres to control levels, the transient parameters were the same as those measured from control fibres. The results in the present paper show that there is a population of morphologically normal *mdx* cells in which the excitation–contraction coupling process is not altered by the lack of dystrophin. This conclusion is supported by a study done by Hollingworth *et al.* (1990) which showed that the charge movement (i.e. the charge generated by the potential sensor during excitation–contraction coupling) was unaltered in the EDL muscles of *mdx* mice compared with that in the EDL muscle of control mice.

The present study shows that the absence of dystrophin from the skeletal muscle tissue of dystrophic *mdx* mice does not, by itself, invariably result in elevated levels of intracellular calcium (Fong *et al.* 1990; Bakker *et al.* 1992).

I acknowledge the expert technical assistance of Ruth I. Cafarella and thank Professor D. George Stephenson for useful discussions and critically reading the manuscript.

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