Titin-Based Modulation of Calcium Sensitivity of Active Tension in Mouse Skinned Cardiac Myocytes

Olivier Cazorla,* Yiming Wu,* Thomas C. Irving, Henk Granzier

Abstract—We studied the effect of titin-based passive force on the length dependence of activation of cardiac myocytes to explore whether titin may play a role in the generation of systolic force. Force-pCa relations were measured at sarcomere lengths (SLs) of 2.0 and 2.3 μm. Passive tension at 2.3 μm SL was varied from ~1 to ~10 mN/mm² by adjusting the characteristics of the stretch imposed on the passive cell before activation. Relative to 2.0 μm SL, the force-pCa curve at 2.3 μm SL and low passive tension showed a leftward shift (ΔpCa50 [change in pCa at half-maximal activation]) of 0.09±0.02 pCa units while at 2.3 μm SL and high passive tension the shift was increased to 0.25±0.03 pCa units. Passive tension also increased ΔpCa50 at reduced interfilament lattice spacing achieved with dextran. We tested whether titin-based passive tension influences the interfilament lattice spacing by measuring the width of the myocyte and by using small-angle x-ray diffraction of mouse left ventricular wall muscle. Cell width and interfilament lattice spacing varied inversely with passive tension, in the presence and absence of dextran. The passive tension effect on length-dependent activation may therefore result from a radial titin-based force that modulates the interfilament lattice spacing. (Circ Res. 2001;88:1028-1035.)

Key Words: x-ray diffraction ■ myofilament lattice ■ collagen ■ Frank-Starling

The precise mechanisms by which the heart is able to enhance its contractile performance in response to an increase in volume (Frank-Starling mechanism) remain to be resolved. The cellular basis of the Frank-Starling mechanism underlies the passive force of cardiac myocytes.9,10 This force I-band segment of titin functions as a molecular spring that muscle, except toward the upper limit of the physiological SL range where collagen dominates.9,11 A passive force–based increase in the number of crossbridges bound to actin has been suggested by earlier work on insect flight muscle12 and rabbit psoas muscle.13 Here we investigated the involvement of titin-based passive tension in the SL dependence of Ca²⁺ activation of skinned cardiac myocytes and in modulating the interfilament lattice spacing. Force-pCa curves were measured at SL 2.0 and 2.3 μm, and the pCa50 (pCa at half-maximal activation, an index of Ca²⁺ sensitivity) was determined at various levels of passive tension achieved by varying the stretch characteristics imposed on the cell before activation. We found that passive tension significantly influences both the length dependence of activation and the myofilament lattice spacing in normal and compressed muscle. Thus, titin is not just a passive spring that is independent of active force development, which is the conventional view, but titin also influences actomyosin interaction, possibly via modulating interfilament lattice spacing.

Materials and Methods

Preparations and Solutions

Myocytes and muscles were isolated from mice and skinned as previously described.11,14 Solution composition was as described, as were all contained protease inhibitors.9 For osmotic compression,
X-Ray Diffraction
The BioCAT undulator-based beamline at the Argonne National Laboratory (Argonne, IL) was used. Muscles were mounted to a force transducer and a motor in a small trough with windows for collection of x-ray patterns and viewing of striations (SL was determined as described above). X-ray patterns were collected and spacings of the 1,0 and 1,1 equatorial reflections were measured and converted to d0,1 values.

Titin Degradation
X-ray experiments were performed on skinned muscle preparations that had been trypsinized (0.25 μg/mL; 25 minutes at 25°C) to degrade titin. To determine the amount of intact titin, preparations were solubilized after completion of the x-ray experiment and analyzed with SDS-PAGE. For details, see References 9 and 16.

Statistics
Results are mean±SE (unless indicated otherwise). Significant differences were assigned using the paired or unpaired Student t test (as appropriate) or 1-way ANOVA and Tukey multiple comparison with P<0.05.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results
The Ca²⁺ sensitivity of active force was studied at various levels of passive force. To determine active force, we subtracted the passive force before activation from total force during activation. However, this is only valid if during activation sarcomeres are isometric, because SL shortening reduces passive force and stretching has the opposite effect. We measured SL online and observed (as have others) that although cells were kept isometric during contraction, sarcomeres typically changed length, especially when activation was maximal. The SL change varied somewhat from cell to cell, and for each cell we first performed a test contraction at pCa 4.5 and only continued with those cells that were well attached with minimal (%0.1 μm) SL changes. When required, cell length was varied during contraction so as to keep SL constant (see Figure 1). The differences in SL between the start and the peak of contraction of all cells used in this study were small (%0.03±0.02 μm). Thus, changes in passive force during contraction are negligible, and active force equals the activation-induced force increase.

**Effects of Passive Tension and Dextran on Calcium Sensitivity**

<table>
<thead>
<tr>
<th>Dextran, %</th>
<th>n</th>
<th>Passive Tension, mN/mm²</th>
<th>pCa50</th>
<th>nH</th>
<th>Passive Tension, mN/mm²</th>
<th>pCa50</th>
<th>nH</th>
<th>ΔpCa50</th>
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</thead>
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<tr>
<td>0</td>
<td>10</td>
<td>0.5±0.1</td>
<td>5.69±0.02</td>
<td>3.1±0.2</td>
<td>1.7±0.3*</td>
<td>5.78±0.03*</td>
<td>3.0±0.2</td>
<td>0.09±0.01*</td>
</tr>
<tr>
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<td>0.6±0.1</td>
<td>5.64±0.02</td>
<td>2.8±0.2</td>
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<td>6.01±0.03</td>
<td>2.9±0.2</td>
<td>0.09±0.01*</td>
</tr>
<tr>
<td>2.5</td>
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<td>6.06±0.03</td>
<td>2.7±0.2</td>
<td>0.15±0.01*</td>
</tr>
</tbody>
</table>

n indicates number of cells; nH, Hill coefficient; ΔpCa50, pCa50 at SL 2.3 minus pCa50 at 2.0 μm (each cell functioned as its own control).

*Significant difference between results at high vs low passive tension.

dextran (T500) was used. For additional details see, online expanded Materials and Methods section available at http://www.circresaha.org.

Experimental Setup
The setup was as described. SL was measured online at 15 Hz (Ionoptix Corp). The system uses a pseudo–2-dimensional FFT analysis of the digitized striation images of the attached cell. SL could be controlled during activation via adjusting the motor input voltage so as to keep SL at a constant value. Force was normalized by the cross-sectional area of the cell. The force-pCa relation of freshly isolated cells was measured at 2.0 μM and then at 2.3 μM SL (15°C). Passive force was varied as explained below. Active forces at submaximal activations were normalized to that produced at pCa 4.5 at the same SL. (Force at pCa 4.5 slightly diminished with the number of imposed contractions; the mean reduction during the measurement of 2 force-pCa curves was 23% [n=41].) The relation between relative force and pCa was fitted to the following equation: force = [Ca²⁺]nH/(K + [Ca²⁺])nH, where nH is the Hill coefficient and pCa,pCa is −(log K)/nH. Values in the Table are the average values from all cells studied under a certain condition. All shown curves were fitted to the average force produced at each pCa.
Passive force was adjusted by varying the SL history before activation. High levels of passive force were obtained by rapidly stretching the cell to 2.3 μm with activation following immediately (Figure 1). Low to intermediate levels of passive force were obtained by (1) stretching sarcomeres to a length that exceeded 2.3 μm (typically to ~2.5 μm), (2) holding cell length constant, (3) releasing to 2.3 μm SL, and (4) holding the cell at 2.3 μm SL then activating. The central panel of Figure 2A shows an example of this protocol. During the hold phase at long SL, passive force rapidly decayed, most likely because of contour length gain of subdomains within the extensible region of titin. Recovery takes place if the cell is completely released to the slack length and held there for several minutes. A partial release is insufficient for full refolding, and passive force will therefore be lower. These protocols allowed us to vary passive tension at SL 2.3 μm between ~1 and ~10 mN/mm².

When cells were submaximally activated at 2.3 μm SL, active force was significantly lower if passive force was low (compare Figure 2A, middle and right). This reduction in active force did not result from protocol-induced damage to either titin or the contractile apparatus, as both passive and active forces recovered after a ~10-minute rest period at the slack length (compare Figure 2A, left and right). To ensure that all contractions were induced at the same passive force level, between contractions cells were always released to their slack length followed by a ~10-minute rest, and then the exact same stretch-activation protocol was imposed.

Force-pCa relations were determined at 2.0 and 2.3 μm SL at high (8.7±0.3 mN/mm²) and low (1.7±0.3 mN/mm²) passive tension. At both passive tension levels, the force-pCa curves at 2.3 μm were shifted leftward relative to the curve at 2.0 μm SL. The shift was significantly larger at high passive tension (Figure 2B and Table). The pCa for half-maximal activation (pCa½) increased by 0.25±0.02 pCa units and 0.09±0.01 pCa units for high and low passive tensions, respectively. Results were independent of the order in which the experiments were performed (high versus low passive tension), indicating that no permanent damage was done by these protocols. We measured the maximal active tension (pCa 4.5) in experiments in which 3 contractions were induced (in random order), as follows: (1) 2.0 μm SL, (2) 2.3 μm SL (low passive tension), and (3) 2.3 μm SL (high passive tension). Maximal active tensions (in mN/mm²) were 30.7±2.3 (n=18), 31.6±1.7 (n=13), and 34.3±1.8 (n=18), respectively. Only results at 2.3 μm SL (high passive tension) were significantly higher than at SL 2.0 μm (ANOVA, P<0.05).

Varying the amplitude and the duration of prestretch resulted in intermediate passive tension levels at SL 2.3 μm. Results from 29 cells were pooled in passive tension bins of 2.0 mN/mm², and their corresponding ΔpCa½ (pCa½ at SL 2.3−pCa½ at 2.0 μm) values were averaged. Figure 2C shows that at passive tensions between 0 and 2 mN/mm², ΔpCa½ is 0.09 pCa units and that at higher tensions ΔpCa½ increases until it reaches 0.25 pCa units at a passive tension of 10 mN/mm². Linear regression (broken line in Figure 2C) shows that the length dependence of activation (ΔpCa½) has a passive tension–independent component (~0.08 pCa units in size) and a component that varies with passive tension.

Considering that the large prestretch/partial release protocol requires stretch to nonphysiological SLs, we also measured the force-pCa relation of cells in which passive tension was reduced with trypsin, as an independent method. Because...
of the high trypsin sensitivity of the PEVK domain of titin, a mild trypsin treatment can be used to specifically degrade the I-band region of titin and to thereby lower passive tension at a given SL10,11 (see also below and online Materials and Methods available at http://www.circresaha.org). Trypsin-treated cells were stretched to SL 2.3 \( \mu \text{m} \) and then immediately activated. Because of degradation of titin, passive tension was now low (1.7 \( \pm \) 0.6 mN/mm\(^2\); \( n = 6 \)). The force-pCa relation so obtained was shifted to the right compared with the curve measured at high passive tension (Figure 2D). The pCa\(_{50} \) (5.79 \( \pm \) 0.04) and maximal active tension (33.1 \( \pm \) 2.8 mN/mm\(^2\)) are indistinguishable from that at low passive tension obtained after the large prestretch/partial release protocol (5.78 \( \pm \) 0.03, and 31.6 \( \pm \) 1.7 mN/mm\(^2\), respectively). Thus, reduction of passive tension via either trypsin treatment or a large stretch/partial release leads to the same force-pCa relation.

We studied whether the passive tension–induced shift of the force-pCa relation involves a passive tension effect on the myofilament lattice spacing as indicated by the cell width at 2.3 \( \mu \text{m} \) SL. Cell width was significantly smaller at high passive tension than at low passive tension (Figure 3C [a]). Furthermore, during stress recovery (Figure 3C [b]), cells shrank significantly.

It is well known that the myofilament lattice spacing of striated muscles expands during skinning as a result of the loss of the osmotic constraint to swelling imposed by the sarcolemma.4,18,19 It has been reported that the myofilament lattice on skinning of cardiac muscle expands 19 and that this may be countered with \( \approx 2.5\% \) (wt/vol) dextran T-500. To test whether passive tension affects the length dependence of activation in conditions in which skinning-induced swelling was compensated, we measured the force-pCa relations at high and low passive tension in the presence of 2.5\% dextran. In the presence of dextran, the cell width at SL 1.9 \( \mu \text{m} \) was reduced by \( \approx 8\% \) (Figure 4A), consistent with the 7\% to 8\% reduction reported by others.6,20 The force-pCa relations at
2.0 μm SL in the presence of dextran were shifted to the left (relative to no dextran) to a position similar to the one obtained without dextran at 2.3 μm SL (Figure 4B and Table). This is consistent with the results of others obtained on cardiac myocytes\(^6\) that showed that dextran sensitizes the cells at short SL. To our knowledge, the effect of dextran on the force-pCa relation of cardiac myocytes at 2.3 μm SL has not been investigated before. Relative to the curve at 2.0 μm SL (with dextran), our experiments revealed a $\Delta pC_{a50}$ of 0.08 pCa units at low passive tension and 0.15 pCa units at high passive tension (Figures 4B and 4C, Table).

We measured the maximal active tension in experiments in which 3 maximal active contractions were induced, at SL 2.0, SL 2.3 (low passive tension), and SL 2.3 (high passive tension) μm, in a randomized order. Maximal active tensions (in mN/mm\(^2\)) were 32.4 ± 2.5 (n = 10), 36.6 ± 2.3 (n = 10), and 34.9 ± 2.0 (n = 10), respectively. Results comparing high versus low passive tension at 2.3 μm were not significantly different. The maximum tensions at 2.3 μm SL (low passive tension) and those at 2.3 μm SL (high passive tension) were both significantly higher than at 2.0 μm.

To further probe the mechanism underlying these findings, we studied the effect of passive tension on myofilament lattice spacing by using low-angle x-ray diffraction. Because x-ray diffraction on cells is currently not feasible, because of their low x-ray scattering mass, for these studies we used mouse skinned myocardium dissected from the left ventricular free wall. Figure 6A, panel 1, shows a typical x-ray pattern from a skinned preparation in relaxing solution with clearly resolved equatorial reflections. (SDS-PAGE showed that titin was unaffected by the x-ray exposure.) The separation of these reflections allowed us to measure lattice spacing with 0.1-nm resolution. Myofilament lattice spacing decreased significantly as SL was increased (Figure 6A), which was consistent with recent measurements on rat cardiac trabeculae.\(^4\)
To vary passive tension, we were unable to use the “large prestretch protocol,” as collagen in mouse myocardium limits the maximal SL with reversible mechanical characteristics to \( \approx 2.3 \, \mu m\). Again, we varied passive tension by degrading titin with trypsin (0.25 \( \mu g \) trypsin/mL, 25 minutes at 25°C). Treating mouse skinned myocardium with trypsin greatly degraded titin \((T_1)\) without significantly affecting other proteins (Figure 5B). To test whether trypsin affects collagen-based force, we treated collagen strips as well as myocardium with trypsin that had been extracted with high salt to abolish titin as a source of passive force. The force of collagen was unaffected by trypsin (Figures 5C and 5E). We also tested whether trypsin affects intermediate filament–based force and found that this force also is not affected by trypsin (Figures 5D and 5E). Finally, we measured the force-pCa relation at a SL of 1.9 \( \mu m\) and found that the relation was unaffected by trypsin (Figure 5F). Neither pCa_{50} (control, 5.71\( \pm 0.03 \, [n = 12]\); trypsin treated, 5.73\( \pm 0.04 \, [n = 12]\)) nor maximal active tension (control, 25.7\( \pm 2.0 \, mN/mm^2 \, [n = 12]\); trypsin treated, 23.3\( \pm 1.7 \, mN/mm^2 \, [n = 12]\)) were significantly affected by trypsin. Thus, under our experimental conditions trypsin specifically abolishes titin-based passive force.

An example of an x-ray diffraction pattern after trypsin treatment is shown in Figure 5A, panel 2, and the SL dependence of the \( d_{1,0}\) spacing before and after trypsin treatment in Figure 6A. Degradation of titin significantly increased the lattice spacing with an average increase of \( \approx 3 \, nm\). The large increase in \( d_{1,0}\) at SL 1.9 \( \mu m\) and our finding that trypsin treatment does not affect calcium sensitivity at SL 1.9 \( \mu m\) (Figure 5F) suggests that calcium sensitivity is independent of \( d_{1,0}\) spacing in the range of 44 to 47 nm. Although support for the idea that changes in myofilament lattice spacing contribute to length-dependent activation is compelling, this finding may be viewed as a cautionary note.

We also studied whether degrading titin had an effect on the osmotically compressed lattice achieved by adding dextran. Definitive studies of the amount of dextran required to restore the in vivo lattice spacing in skinned cardiac muscle have not been reported; earlier studies used a range from 2% to 4% dextran, and we chose 4% for these experiments. An example of an x-ray pattern is shown in Figure 5A, panel 3, and the SL dependence of \( d_{1,0}\) in Figure 6B. Results indicate that at a given SL, dextran greatly reduces the lattice spacing. For example, at SL 2.1 \( \mu m\) dextran reduced \( d_{1,0}\) from 42.4 to 35.4 \( nm\). (Note that this spacing in dextran is close to that of intact cardiac muscle at SL 2.1 \( \mu m\); for rat, reported values are 34.8 and 35.6 \( nm\).) The effect of degrading titin with trypsin in the presence of dextran was then studied. An example of an x-ray pattern is shown in Figure 5A, panel 4, and measurements are shown in Figure 6B. Degrading titin significantly increased the lattice spacing, this increase being largest at 1.9 \( \mu m\) SL with a gradual decrease with SL. These studies support the notion that titin modulates the interfilament lattice spacing in both the presence and the absence of dextran.

**Discussion**

Numerous studies have shown that an important component of the Frank-Starling mechanism is the length dependence of the calcium sensitivity of force. A now widely held explanation for length-dependent activation is that the change in interfilament spacing that accompanies SL change modulates the probability of actomyosin interaction at the same calcium concentration. The mechanism by which interfilament spacing affects actomyosin interaction may involve an interfilament-spacing effect on weakly bound crossbridges by affecting their number or the rate of transition from the weak to strong binding states. Here we report that titin modulates interfilament spacing and that titin-based passive tension influences length-dependent activation.

**Effect of Titin-Based Passive Tension on Calcium Sensitivity**

Because of stress relaxation, titin-based passive tension at a given SL is not constant but decreases with time. We took advantage of the high degree of passive stress relaxation at long SL and its slow recovery on partially releasing the cell, to vary passive tension at a SL of 2.3 \( \mu m\) and study its effect of the force-pCa relation. Results indicate that the length dependence of activation (\( \Delta pCa_{50}\)) has a passive tension–independent component of 0.08 pCa units (intercept of line in Figure 2C) that is likely to involve thin- and thick-filament–based processes independent of titin. The passive tension–independent component is somewhat less than the \( \approx 0.12 \, \Delta pCa_{50}\) values reported by other laboratories studying the mouse. Although passive tension was not reported in these previous studies, they used protocols generally consisting of a slow stretch followed by a long wait period before activation, and passive tensions are therefore likely to have been relatively low (our Figure 2C indicates that a \( \Delta pCa_{50}\) of 0.12 is accompanied by \( \approx 2.5 \, mN/mm^2\) passive tension). Thus, the \( \Delta pCa_{50}\) values at low passive tension found here are in general agreement with those of others. High passive tensions were achieved by rapidly stretching cells to SL 2.3 \( \mu m\) and then immediately activating them (Figure 1). We found that passive tension significantly enhances the length dependence of activation with a \( \Delta pCa_{50}\) of 0.25 pCa units at the highest passive tensions used. Thus, for maximal calcium sensitivity of skinned cardiac myocytes, a high level of titin-based passive tension is required.

**Effect of Titin on Interfilament Spacing**

For cardiac myocytes, we used the cell width as an indicator of interfilament spacing. Although cell width is an imperfect indicator, it nevertheless provides qualitative insights into myofilament lattice behavior. We found that passive tension correlates negatively with cell width (Figure 3), suggesting that titin modulates myofilament lattice spacing. In agreement with this are the low-angle x-ray diffraction studies on cardiac muscle that showed that degradation of titin significantly increases \( d_{1,0}\). These findings are consistent with results on mechanically skinned skeletal muscle fibers in which a close correlation is found between titin-based passive tension and \( d_{1,0}\) and in which, after degradation of titin, \( d_{1,0}\) is found to be independent of SL. Thus, titin is a modulator of interfilament lattice spacing in skeletal and cardiac muscle.

After titin degradation, the myofilament lattice was still responsive to SL (Figure 6). This suggests that in addition to...
titin, other modulators of myofilament lattice spacing exist. A possible candidate is collagen. By comparing $d_{1,0}$ of chemically and mechanically skinned skeletal muscle fibers, it has been shown that at long SL collagen compresses the myofilament lattice in skeletal muscle. In myocardium, collagen is the main source of passive tension at long SL, whereas titin is the main source at short SL. If collagen and titin both affect lattice spacing, eliminating the force of titin will have the largest effect on spacing at short SL and the smallest at long SL, giving rise to steeper $d_{1,0}$–SL relations after elimination of titin. This expectation is in agreement with our findings (Figure 6) and supports the idea that the myofilament lattice spacing is under the influence of both collagen and titin.

The segment of titin near the Z line binds strongly to the thin filament and at the A/I junction to the thick filament tip. Thus, the extensible region of titin is not parallel with the filaments, and the force ($F$) of titin has a longitudinal and radial component ($F_L$ and $F_r$). B, Radial force–SL relation and interfilament electrostatic repulsive force ($F_{ES}$)–SL relation of titin. For details see text and online expanded Materials and Methods section available at http://www.circresaha.org.

Figure 7. Model of titin-based myofilament lattice modulation. A, Near the Z line, titin binds the thin filament and at the A/I junction to the thick filament tip. Thus, the extensible region of titin is not parallel with the filaments, and the force ($F$) of titin has a longitudinal and radial component ($F_L$ and $F_r$). The segment of titin near the Z line binds strongly to the thin filament and at the A/I junction to the thick filament tip. Thus, the extensible region of titin is not parallel with the filaments, and the force ($F$) of titin has a longitudinal and radial component ($F_L$ and $F_r$). As a result, titin is expected to develop a longitudinal and radial component ($F_L$ and $F_r$) of titin.

In conclusion, titin influences the length dependence of calcium sensitivity of active force in cardiac myocytes, and the underlying mechanism may involve an effect of titin on myofilament lattice spacing. These findings challenge the conventional notion that titin is independent of actomyosin interaction and an increase in calcium sensitivity. The notion that the crossbridge order can vary in passive muscle is supported by studies that investigated thick filament structure in response to changes in passive stretch, temperature, phosphorylation of myosin light chains, and C protein. Thus, thick filament strain as well as interfilament spacing may be involved in linking titin to calcium sensitivity, and their relative importance and interrelationship remains to be established.

In conclusion, titin influences the length dependence of calcium sensitivity of active force in cardiac myocytes, and the underlying mechanism may involve an effect of titin on myofilament lattice spacing. These findings challenge the conventional notion that titin is independent of actomyosin interaction, and they suggest that titin has the potential to enhance systolic performance as the ventricular volume is increased.

Acknowledgments

This work was supported by National Institutes of Health (NIH) Grants HL62774 and HL62881 (to H.G.), American Heart Association (AHA) Grant 9950459N (to T.C.I.), and an AHA fellowship (to O.C.). Use of Advanced Photon Source was supported by the Department of Energy (Grant W-31-109-ENG-38). BioCAT is an NIH-supported research center (Grant RR08630). We acknowledge Dr P.P. de Tombe and laboratory members for assistance and use of their mechanics and setup for x-ray work and Drs S. Labeit, K. Campbell, B. Slinker, and P.P. de Tombe for discussions. We acknowledge Jennifer Eldridge for gel electrophoresis.

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Titin and Length Dependence of Activation

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_Circ Res._ 2001;88:1028-1035; originally published online May 10, 2001; doi: 10.1161/hh1001.090876

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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