Properties of Na\(^+\) currents conducted by a skeletal muscle L-type Ca\(^{2+}\) channel pore mutant (SkEIIIK)

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Abbreviations: EC, excitation-contraction; TEA, tetraethylammonium

Four glutamate residues residing at corresponding positions within the four conserved membrane-spanning repeat of L-type Ca\(^{2+}\) channels are important structural determinants for the passage of Ca\(^{2+}\) across the selectivity filter. Mutation of the critical glutamate in Repeat III in the \(\alpha_{1s}\) subunit of the skeletal L-type channel (Ca\(_{1.1}\)) to lysine virtually eliminates passage of Ca\(^{2+}\) during step depolarizations. In this study, we examined the ability of this mutant Ca\(_{1.1}\) channel (SkEIIIK) to conduct inward Na\(^+\) current. When 150 mM Na\(^+\) was present as the sole monovalent cation in the bath solution, dysgenic (Ca\(_{1.1}\) null) myotubes expressing SkEIIIK displayed slowly-activating, non-inactivating, nifedipine-sensitive inward currents with a reversal potential (45.6 ± 2.5 mV) near that expected for Na\(^+\). Ca\(^{2+}\) block of SkEIIIK-mediated Na\(^+\) current was revealed by the substantial enhancement of Na\(^+\) current amplitude after reduction of Ca\(^{2+}\) in the external recording solution from 10 mM to near physiological 1 mM. Inward SkEIIIK-mediated currents were potentiated by either ±Bay K 8644 (10 \(\mu\)M) or 200-ms depolarizing prepulses to +90 mV. In contrast, outward monovalent currents were reduced by ±Bay K 8644 and were unaffected by strong depolarization, indicating a preferential potentiation of inward Na\(^+\) currents through the mutant Ca\(_{1.1}\) channel. Taken together, our results show that SkEIIIK functions as a non-inactivating, junctionally-targeted Na\(^+\) channel when Na\(^+\) is the sole monovalent cation present and urge caution when interpreting the impact of mutations designed to ablate Ca\(^{2+}\) permeability mediated by Ca\(_{v}\) channels on physiological processes that extend beyond channel gating and permeability.

Introduction

The pore-forming \(\alpha_{1s}\) subunits of voltage-gated Na\(^+\) (Na\(_{v}\)) and Ca\(^{2+}\) (Ca\(_{v}\)) channels are composed of four conserved domains consisting of six transmembrane segments (S1-S6) each, which are joined by three relatively non-conserved cytoplasmic linker regions.\(^{1,4}\) The S5-S6 linkers of domains I-IV make structural contributions to the selectivity filter that ultimately determines which ions pass through the channel pore. In the case of Na\(_{v}\) channels, a highly-conserved D-E-K-A motif formed by the convergence of the four S5-S6 linkers facilitates the passage of Na\(^+\),\(^{5,7}\) while the preference of Ca\(_{v}\) channels for Ca\(^{2+}\) relies on an E-E-E-E motif in the corresponding positions.\(^{5,7}\)

Substitution of a lysine for the critical glutamate in Repeat III in the \(\alpha_{1s}\) subunit of the skeletal L-type channel (Ca\(_{1.1}\)) to lysine virtually eliminates the ability of the channel to conduct inward Ca\(^{2+}\) or Ba\(^{2+}\) currents, but still allows for passage of Li\(^+\).\(^{7,8}\) The limited Ca\(^{2+}\) permeability of this Ca\(_{1.2}\) pore mutant (heretofofore referred to as CEIIIK, as in ref. 9) enabled the investigation of the effects of 1,4-dihydropyridine agonists and/or strong depolarization on both inward (divalent) and outward (monovalent) currents.\(^{10}\) Like the wild-type Ca\(_{1.2}\) channel,\(^{11}\) both of these experimental maneuvers augmented inward tail currents upon repolarization.

In contrast, outward currents via CEIIIK were either little affected by strong depolarization or reduced by dihydropyridine agonists, revealing inward rectification of the potentiated state of the channel.

The skeletal muscle Ca\(_{1.1}\) channel isoform is highly conserved with Ca\(_{1.2}\), particularly within the four transmembrane regions.\(^{1,2}\) Ca\(_{1.1}\) serves dual functions both as the voltage sensor for excitation-contraction (EC) coupling and as an L-type Ca\(^{2+}\) channel (reviewed in ref. 12). Since Ca\(^{2+}\) entry via Ca\(_{1.1}\) is not required to engage EC coupling,\(^{13,14}\) introduction of the glutamate to lysine mutation in Repeat III into Ca\(_{1.1}\) has no obvious effect on the ability of the mutant Ca\(_{1.1}\) channel (SkEIIIK) to restore EC coupling when expressed in dysgenic (Ca\(_{1.1}\) null) myotubes.\(^{9,15,16}\) Despite the persistence of voltage-sensing activity, SkEIIIK is virtually impermeant to divalent cations\(^{9,15}\) unless potentiated simultaneously by strong depolarization and application of the dihydropyridine agonist ±Bay K 8644.\(^{16,17}\) However, the ability of SkEIIIK to conduct monovalent cations has not been investigated previously. Here, we demonstrate that SkEIIIK conducts slowly-activating, non-inactivating inward Na\(^+\) currents in the presence of near physiological Na\(^+\) and we exploit this inward current to probe the permeation properties of Ca\(_{v}\)1.1.
Results

SkEIIIK conducts Na+. Figure 1A shows currents recorded from a dysgenic (Ca1.1 null) myotube expressing the Ca1.1 pore mutant SkEIIIK with 150 mM tetraethylammonium (TEA+) and 10 mM Ca2+ present in the external recording solution. As demonstrated previously in references 9, 15 and 16, no inward currents were observed under these experimental conditions. However, outward currents carried by Cs+ included in the internal recording solution were evoked by test potentials >20 mV. Upon replacing the 150 mM external TEA+ with either 150 mM Na+ (A) or 150 mM Na+ (B) as the sole monovalent cation present in the external recording solution (see Materials and Methods). (C) I/V relationships for dysgenic myotubes expressing SkEIIIK in the presence of TEA+ (●; n = 6) or Na+ (○; n = 24). Currents were evoked at 0.1 Hz by 200-ms test depolarizations ranging from -40 mV through +70 mV in 10 mV increments, after a prepulse protocol (see Materials and Methods). (D) Inward Na+ current recorded before (1) and 230 seconds after addition (2) of 10 μM nifedipine to the bath (test potential = +30 mV; linear cell capacitance = 337 pF). Throughout, error bars represent ±SEM.

Figure 1. SkEIIIK conducts Na+. Current families elicited by 200-ms depolarizations from -50 mV to 0, 20, 40 and 60 mV are shown for dysgenic myotubes expressing SkEIIIK with either 150 mM TEA+ (A) or 150 mM Na+ (B) as the sole monovalent cation present in the external recording solution (see Materials and Methods). (C) I/V relationships for dysgenic myotubes expressing SkEIIIK in the presence of TEA+ (●; n = 6) or Na+ (○; n = 24). Currents were evoked at 0.1 Hz by 200-ms test depolarizations ranging from -40 mV through +70 mV in 10 mV increments, after a prepulse protocol (see Materials and Methods). (D) Inward Na+ current recorded before (1) and 230 seconds after addition (2) of 10 μM nifedipine to the bath (test potential = +30 mV; linear cell capacitance = 337 pF). Throughout, error bars represent ±SEM.

L-type channel antagonist nifedipine (10 μM) reduced the slow inward Na+ current by 81 ± 4% (n = 4; Fig. 1D). Importantly, the reversal potential of the SkEIIIK-mediated current was 45.6 ± 2.5 mV (n = 24; compare with 89 ± 2.2 mV for 13 dysgenic myotubes expressing GFP-Ca1.1 in 150 mM TEA+/10 mM Ca2+; data not shown), consistent with the idea the slow inward current was predominantly carried by Na+ with little or no contribution from Ca2+ present in the external recording solution (Fig. 1C). Still, it is important to note that outward driving force on Cs+ also contributes to the observed reversal potential.

Ca2+ block of SkEIIIK-mediated Na+ currents. Passage of Ca2+ through the selectivity filter of L-type Ca2+ channels is dependent on the presence of four glutamate residues within the P loop of each conserved repeat. These glutamates are thought to support two high-affinity Ca2+ binding sites in which binding of one Ca2+ to one site is thought to facilitate the passage of the other bound Ca2+ through the pore. In the context of this model, disruption of one of the Ca2+ binding sites by substitution of a lysine residue for the conserved glutamate in Repeat III of the cardiac α1C L-type channel subunit attenuates the ability of the pore to accommodate two Ca2+ ions, with the result that the channel permits only monovalent cation flux that is antagonized by the presence of extracellular Ca2+. In order to investigate whether Na+ current conducted by SkEIIIK was also subject to block by Ca2+, the concentration of Ca2+ in the external solution was reduced from 10 mM to 1 mM. As depicted in Figure 2A, this experimental maneuver resulted in substantial enhancement of SkEIIIK-mediated Na+ currents. Although a hyperpolarizing shift in the voltage dependence of activation (-14 mV; n = 8; p < 0.005; paired t-test) may have contributed to the increase in SkEIIIK-mediated Na+ current at less depolarized test potentials, the removal of Ca2+ block also appeared to be important because inward Na+ currents recorded in 1 mM Ca2+ were greater at every test depolarization than those recorded in 10 mM Ca2+ in the same cells immediately before solution exchange (Fig. 2B).

Effects of ±Bay K 8644 on SkEIIIK-mediated currents. Figure 3A shows SkEIIIK-mediated currents recorded following acute exposure to the dihydropyridine agonist ±Bay K 8644 (10 μM). In these experiments, ±Bay K 8644 in paired experiments had little effect on the peak amplitude of the inward Na+ current via SkEIIIK (n = 9; p > 0.05; Fig. 3B). The reduced sensitivity of inward step current to ±Bay K 8644 relative to the wild-type channel21,22 was most likely a consequence of Cs+ competing with Na+ for permeation. In contrast to its relatively minor effect on the inward Na+ current evoked by 200-ms step depolarizations, exposure to ±Bay K 8644 decreased...
SkEIIIK-mediated outward monovalent currents (Fig. 3A and B); outward currents evoked by stepping from -50 mV to +70 mV were reduced from 10.9 ± 1.9 to 4.4 ± 1.6 pA/pF by exposure to ±Bay K 8644 (n = 9; p < 0.005, paired t-test). Accompanying this reduction in outward current, there was a depolarizing shift in the reversal potential (~7 mV; p < 0.005, paired t-test; Fig. 3B and Table 1). This shift in reversal potential is suggestive that ±Bay K 8644 causes the channel to have altered permeation properties, an idea which is investigated more completely in the following section (see below).

In contrast, to the diverse effects of ±Bay K 8644 on the currents during 200-ms depolarizing steps, its effects on tail currents were similar to those previously described for wild-type Cav1.1 whole-cell currents, increasing the amplitude and slowing the decay (Fig. 3A and compare with 1B). In particular, for tail currents elicited by repolarization from +40 mV to -50 mV, ±Bay K 8644 enhanced inward tail current amplitude by 60 ± 21% and increased the decay time constant by 272 ± 83% (Fig. 3C; n = 8; p < 0.05 and < 0.005, respectively; paired t-tests). Both these effects are consistent with single channel studies of L-type channels which have shown that ±Bay K 8644 causes the channel to enter a potentiated state (i.e., mode 2 gating) with higher Po and slower deactivation.26,27

**Preferential potentiation of inward SkEIIIK-mediated Na+ current.** As described above, the ±Bay K 8644-induced Na+ current is sensitive to ±Bay K 8644. (A) Representative currents from a dysgenic myotube expressing SkEIIIK in the presence of ±Bay K 8644 (10 μM). Currents shown were elicited by 200-ms depolarizations from -50 mV to test potentials ranging from 0 mV to +70 mV after a prepulse protocol. (B) Peak SkEIIIK-mediated currents during 200-ms depolarizations are plotted as a function of test potential before (●) and after (○) exposure to 10 μM ±Bay K 8644. (C) Effect of ±Bay K 8644 on the amplitude (left) and deactivation time constant (right) of SkEIIIK tail currents recorded upon repolarization from +40 mV to -50 mV (n = 8 paired cells). Deactivation was fit by Eqn. 1. Asterisks indicate significant differences between control and ±Bay K 8644-treated cells (*p < 0.05; **p < 0.01; ***p < 0.005, paired t-test).
Table 1. Reversal potentials

| External recording solution | \( V_{rev} \) (mV) | \( V_{rev} \) (mV) |
|-----------------------------|---------------------|---------------------|
| Na\(^+\)/10 mM Ca\(^{2+}\) (pooled controls) | 45.6 ± 2.5 (24) | N/A |
| TEA\(^-\)/10 mM Ca\(^{2+}\) | ~25.0 (6) | N/A |
| Na\(^+\)/1 mM Ca\(^{2+}\) | 53.8 ± 3.0 (8) | 470 ± 6.9 |
| Na\(^+\)/10 \( \mu \)M ± Bay K 8644 | 51.5 ± 3.6 (9) | 44.6 ± 2.7*** |

Data obtained from dysgenic myotubes expressing SkEIIIK are given as mean ± SEM, with the numbers in parentheses indicating the number of myotubes tested. Reversal potentials were calculated with Eqn. 2 (see Materials and Methods), with the exception of that for TEA\(^-\)/10 mM Ca\(^{2+}\) which was the estimated (\( \pm \)) voltage for development of outward monovalent current (Fig. 1C). Whenever otherwise noted, the external recording solution contained 10 mM Ca\(^{2+}\). Paired controls were recorded in Na\(^+\)/10 mM Ca\(^{2+}\) before replacing this standard external recording with Na\(^+\)/1 mM Ca\(^{2+}\) or Na\(^+\)/10 \( \mu \)M ± Bay K 8644. The asterisks ***, indicate a significant difference (\( p < 0.005 \), paired t-test) relative to the matched control group. N/A = not applicable.

either +60 or +90 mV, inward Na\(^+\) currents were measured upon repolarization to -20 mV (Fig. 4A), and outward currents upon clamping to +60 mV (Fig. 4B). As shown at the bottom of Figure 4A, inward SkEIIIK-mediated tail current carried by Na\(^+\) was potentiated following the depolarization to +90 mV compared to that following the +60 mV pulse. By contrast, the outward current at +60 mV was not measurably potentiated by the prior depolarization to +90 mV (Fig. 4B bottom).

Because the inward tail current decayed fairly rapidly even for a repolarization to -20 mV, we also examined depolarization-induced potentiation in the presence of ±Bay K 8644 (10 \( \mu \)M). As shown in Figure 4C, ±Bay K 8644 greatly increased the amplitude and half-decay time of tail current relative to the currents recorded before ±Bay K 8644 (Fig. 4A). Thus, the potentiation of inward Na\(^+\) current by the prior depolarization to +90 mV was more obvious in the presence of ±Bay K 8644 (Fig. 4C). For outward monovalent current at +60 mV, prior depolarization to +90 mV in the absence of ±Bay K 8644 failed to cause potentiation (Fig. 4D), just as was observed in the absence of the agonist (Fig. 4B).

To quantify the effects of depolarization to +90 mV, we used a “potentiation ratio” of \((I_{p, +90 mV})/(I_{p, +60 mV})\) calculated for both the inward tail current at -20 mV and for the outward current at +60 mV (Fig. 4E). On average, inward tail currents were strongly potentiated by the 200-ms depolarization to +90 mV both in the absence and presence of ±Bay K 8644 (potentiation ratios of 1.85 ± 0.18 and 1.75 ± 0.08, respectively, \( n = 5 \) paired cells). By contrast, the 200-ms depolarization to +90 mV had essentially no effect on outward current at +60 mV either in the absence or presence of ±Bay K 8644 (potentiation ratios of 1.03 ± 0.01 and 1.07 ± 0.08, respectively, \( n = 5 \) paired cells).

Discussion

In this study, we examined the ability of a Ca\(^{1.1}\) pore mutant (SkEIIIK) to conduct inward Na\(^+\) current. When 150 mM Na\(^+\) was present as the sole monovalent cation in the external solution, dysgenic myotubes expressing SkEIIIK displayed nifedipine-sensitive, slowly-activating, non-inactivating inward currents with a reversal potential (-46 mV) near that expected for Na\(^+\) (Fig. 1). Ca\(^{2+}\) block of SkEIIIK-mediated Na\(^+\) currents was revealed by the substantial enhancement of Na\(^+\) current amplitude after reduction of Ca\(^{2+}\) in the external recording solution from 10 mM to 1 mM (Fig. 2). Inward SkEIIIK-mediated Na\(^+\) tail currents were potentiated by ±Bay K 8644 or strong depolarization (Figs. 3 and 4). In contrast, neither ±Bay K 8644 nor strong depolarization enhanced outward monovalent currents conducted by SkEIIIK (Fig. 4).

Previous work with the cardiac channel bearing a lysine substitution corresponding to that of SkEIIIK (CEIIIK, as in ref. 9) has shown that this mutant channel readily conducts inward monovalent cation currents during step depolarizations.\(^{6-8}\) Additionally, CEIIIK produces small inward step Ca\(^{2+}\) currents but SkEIIIK does not.\(^{9}\) Given this difference, it was important to determine whether SkEIIIK also differed from CEIIIK in its ability, or lack thereof, to produce inward step monovalent cation currents. Here, we have shown that SkEIIIK functions as a non-inactivating, junctionally-targeted Na\(^+\) channel when Na\(^+\) is the sole monovalent cation present in the bath (Fig. 1).

Ca\(^{2+}\) block of SkEIIIK-mediated Na\(^+\) current was not unexpected since Yang et al. previously demonstrated that Ca\(^{2+}\) competed with Li\(^+\) for binding sites within the pore of CEIIIK and other Ca\(^{1.2}\) pore mutants. The enhancement of Na\(^+\) current in the present study by the reduction of extracellular Ca\(^{2+}\) from 10 mM to 1 mM lends support to the idea that Na\(^+\) and Ca\(^{2+}\) also compete for sites within the altered selectively filter of the mutant skeletal channel (Fig. 2). Within the context of the current model for Ca\(^+\) channel permeation,\(^{24}\) observed block of the Na\(^+\) current is brought about by a single impermeant Ca\(^{2+}\) lodged in the selectivity filter. Even so, it was important to determine whether SkEIIIK could conduct Na\(^+\) current with 150 mM Na\(^+\)/1 mM Ca\(^{2+}\) in the bath because near physiological levels of external Na\(^+\) and Ca\(^{2+}\) are often used in imaging experiments designed to examine divalent cation fluctuations (see below).

Previously, Leuranguer et al. demonstrated for two Ca\(^{1.2}\) pore mutants, “E2A/E4A-\(\alpha_{1c}\)” and “E3K-\(\alpha_{1c}\)”, that inward Ca\(^{2+}\) currents, but not outward monovalent currents, are potentiated by either strong depolarization or dihydropyridine agonists. It has been difficult to determine whether SkEIIIK demonstrates similar behavior because of its very low Ca\(^{2+}\) permeability. Here, the appreciable inward Na\(^+\) currents that we observed for SkEIIIK made it possible to investigate whether SkEIIIK demonstrates potentiation of inward monovalent cation currents, but not outward ones. Indeed, we found that this is the case (Figs. 3 and 4), but it should be noted that even though the inward tail current for SkEIIIK was predominantly carried by Na\(^+\), there may also have been a small contribution from Ca\(^{2+}\). In particular, we have previously shown that in the presence of TEA\(^+\) the combination of ±Bay K 8644 and strong depolarization puts the channel in a state such that it produces an inward Ca\(^{2+}\) tail current.\(^{16}\) However, the total charge transfer by Ca\(^{2+}\) following repolarization from +80 mV to -50 mV (33 ± 8 nC/\(\mu\)F, \( n = 10 \); please see Fig. 8 of ref. 16) in that study was much smaller.
to the S5-S6 linker mutants described above, a IS6 L775P mutant of Cav1.2 has been used to analyze voltage-dependent catecholamine secretion from bovine chromaffin cells. Although Cav1.2(L775P) was shown not to conduct inward monovalent (i.e., Li⁺) current in the presence of Ca²⁺ at relatively hyperpolarized test potentials, entry of the mutant channel into the potentiated (i.e., mode 2) state may alter its permeation characteristics. Thus, while the use of mutant Ca²⁺ channels is of value in studying Ca²⁺ signaling, it is also important to recognize that such mutant channels may produce inward Na⁺ currents, particularly during prolonged K⁺-induced depolarizations. Such Na⁺ entry could potentially induce Ca²⁺ entry via reverse-mode Na⁺/Ca²⁺ exchange. In this regard, Cao et al. showed that cytoplasmic Ca²⁺ levels were unaffected by prolonged exposure to elevated K⁺, and we found that Ca²⁺ entry could not be detected in dysgenic myotubes expressing SkEIIIK. However, appreciable local changes in intracellular Na⁺ could alter function in ways that would be more difficult to detect than secondary changes in Ca²⁺. Thus, care must be exercised in drawing inferences about Ca²⁺ permeation-independent signaling functions of Cav from channels bearing mutations designed to ablate this Ca²⁺ permeability.

**Materials and Methods**

**Myotube culture and cDNA expression.** All procedures involving mice were approved by the University of Colorado-Anschutz...
Medical Campus Institutional Animal Care and Use Committee. Primary cultures of dysgenic (mdg/mdg) myotubes were prepared as described previously in reference 18. Cultures were grown for 6–7 days in a humidified 37°C incubator with 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM; #15-017-CM, Mediatech), supplemented with 10% fetal bovine serum/10% horse serum (HyClone Laboratories). This medium was then replaced with differentiation medium (DMEM supplemented with 2% horse serum). Two to three days later, single nuclei of dysgenic myotubes were microinjected with a solution of plasmid cDNA encoding SkEIIIK9 (400 ng/μl) and pEYFP-C1 (5–10 ng/μl; Clontech). Fluorescent myotubes were used in experiments two days after microinjection.

Measurement of ionic currents. Pipettes were fabricated from borosilicate glass and had resistances of ~2.0 MΩ when filled with internal solution, which consisted of (mM): 140 Cs-aspartate, 10 Cs2-EGTA, 5 MgCl2 and 10 HEPES, pH 7.4 with CsOH; all myotubes were dialyzed for >5 minutes prior to the current recording. The standard external solution contained (mM): 145 NaCl, 10 CaCl2, 0.002 tetrodotoxin and 10 HEPES, pH 7.4 with NaOH (yielding a total Na+ concentration of ~150 mM). In some experiments, the external CaCl2 was reduced in this solution to 1 mM. In other experiments, 145 mM TEA-Cl replaced the 145 mM NaCl in the standard solution (pH 7.4 with TEA-OH). Test currents were corrected for linear components of leak and capacitive current by either -P/4 or P/4 subtraction. Electronic compensation was used to reduce the effective series resistance (usually to <1 MΩ) and the time constant for charging the linear cell capacitance (usually to <0.5 ms). Ionic currents were filtered at 2 kHz and digitized at 5–10 kHz. In all experiments, a 1-s prepulse to -20 mV followed by a 25-ms repolarization to -50 mV was administered before the test pulse (prepulse protocol),29 to reduce inward currents via endogenous Na+ and Ca2+ channels. Cell capacitance (255 ± 16 pF; n = 36) was determined by integration of a transient from -80 mV to -70 mV using Clampexp 8.0 and was used to normalize current amplitudes (pA/pF). The deactivation phase of SkEIIIK tail currents was fitted as:

I(t) = A [exp(-t/τ) + C] 

(Eqn. 1)

where I(t) is the current at time t after the repolarization, A is the peak tail current amplitude, τ is the deactivation time constant, and C represents the steady current (ref. 11). In some cases, tail current decay was quantified by calculation of ½ time of decay because these data were not fit well by Equation 1. Reversal potentials in individual experiments were calculated using the equation:

V_{rev} = [(I_2 x V_1) - (I_1 x V_2)]/(I_2 - I_1) 

(Eqn. 2)

where I1 and V1 are the inward current and test potential, respectively, just prior current reversal and I2 and V2 are the outward current and test potential, respectively after current reversal. All experiments were performed at room temperature (~25°C).

Pharmacology. Nifedipine (#481981, Calbiochem) was dissolved in 100% EtOH to make a 10 mM stock solution. Racemic Bay K 8644 (kindly supplied by Dr. A. Scriabine, Miles Laboratories, Inc.) was stored as a 20 mM stock in 50% EtOH. Dihydropyridines were stored and used in the dark.

Analysis. Figures were made using the software program SigmaPlot (version 11.0, SSPS Inc.). All data are presented as mean ± SEM. Statistical comparisons were made by paired t-test or by unpaired, two-tailed t-test (as appropriate), with p < 0.05 considered significant.

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