Effect of angiotensin II type 1 receptor on delayed rectifier potassium current in catecholaminergic CATH.a cells

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ABSTRACT

AIM: To study the modulatory effects of angiotensin II (Ang II) on the delayed rectifier potassium (Kv) current (I_{Kv}) and its underlying intracellular mechanism in the catecholaminergic system of rats. **METHODS:** AT_1 and AT_2 receptors of the differentiated and undifferentiated CATH.a cells were determined by radioligands binding assay. The I_{Kv} was recorded with the whole cell patch-clamp configuration in voltage clamp mode on CATH.a cells. **RESULTS:** The Ang II receptor proteins including AT_1 and AT_2 receptors were expressed in CATH.a cells, and the number of the former was significantly more than the latter ($P<0.01$). The I_{Kv} of CATH.a cells was reduced by superfusion with the Ang II (100 nmol/L) ($P<0.05$) in the presence of the AT_2 receptor antagonist PD123319, but was not affected by only superfusion with PD123319. The effect of Ang II on I_{Kv} in CATH.a cells was completely inhibited by addition of AT_1 receptor antagonist losartan. Superfusion with Ang II (100 nmol/L) plus U73122, an inhibitor of phospholipase C (PLC) in the presence of PD123319 had no effect on the I_{Kv} [(20.2±2.8) pA/pF]. Ang II-induced reduction of I_{Kv} was attenuated ($P<0.05$) but not abolished by PKC inhibitor calphostin C (Cal) and selective CaMK II inhibitor KN-93 (10 μmol/L) respectively. However, I_{Kv} reduction was completely abolished by superfusion with both Cal and KN-93. **CONCLUSION:** The inhibition of Kv currents in CATH.a cells by Ang II is mediated by AT_1 receptor, and the PLC, PKC and CaMK II may be involved in signal transduction of AT_1 receptor. The differentiated CATH.a cell is a useful cell model to study Ang II receptor-mediated functional modulation of catecholaminergic system.

INTRODUCTION

Angiotensin II (Ang II) plays an important role in the central regulation of blood pressure[1-3]. The effect of Ang II is initiated by activation of AT_1 receptor and involves the stimulation of catecholaminergic system[4]. Immunostaining studies with special antibody indicated that the type 1 (AT_1) of Ang II reeceptor existed on the catecholaminergic neurons of the rat medulla oblongata[5]. The administration of Ang II into rat brain increases the concentrations of dopamine, epinephrine, and norepinephrine in hypothalamus and brainstem[6,7]. Anatomical and functional evidences support that there is an interaction between the brain angiotensin and central catecholamine systems[1,8]. However, the functional studies of active cells are affected by a lot of biological factors *in vivo*, and can not open out the intracellular mechanisms. The underlying interactions among the intracellular molecules in catecholaminergic neurons are still not fully understood.

CATH.a cell line has been proved to be catecholaminergic cells and having neuron-like characteristics,

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which is derived from tyrosine hydroxylase-positive tumors that developed in the brainstem of a transgenic mouse carrying the SV 40 T-antigen oncogene[9]. The CATH.a cells possess lots of the phenotypic properties of noradrenergic neurons of catecholaminergic nuclei in brain including tyrosine hydroxylase (TH), dopamine-β hydroxylase (DBH), productions of dopamine (D) and norepinephrine (NE), and also expressed several markers of differentiated neurons including filament proteins and Kv, Na^+, and Ca^{2+} channels, and also exhibited functional signaling responses to corticotrophin-releasing factor, vasoactive intestinal peptide, bradykinin, and muscarinic acetylcholine and α_2-adrenergic receptor agonists[10,11]. These data indicate that the CATH.a cell line may be an appropriate system to study the mechanism of action of Ang II on brainstem, hypothalamus or catecholaminergic neurons *in vitro*.

It has been demonstrated that the development of action potential in neurons is involved in delayed rectifier potassium (Kv) channel current (I_{Kv}). Previous studies in primary co-cultured neurons from brainstem and hypothalamus of rats showed that decrease of Kv channel activity or I_{Kv} in some extent resulted in neuronal firing, and that Ang II increased the firing rate of the neurons in different extent in presence of AT_2 receptor antagonist PD123319. It is indicated that Ang II decreases I_{Kv}, and this effect could be mediated by AT_1 receptor and by activation of protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (CaM KII)[12]. We have detected expression of Ang II receptors, AT_1 and AT_2, in the CATH.a cells by RT-PCR. The aim of the study was to investigate the expression of AT_1 and AT_2 receptors and the intracellular mechanisms of Ang II decreasing I_{Kv} on the CATH.a cells *in vitro*.

MATERIALS AND METHODS

CATH.a cell culture CATH.a cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA), and cultured in the following protocol provided by ATCC. CATH.a cells were planted onto poly-*L*-lysine-precoated 35-mm Nunc plastic tissue culture dishes and grown in medium containing RPMI-1640 supplemented with 8 % horse serum, 4 % fetal bovine serum, and 1 % penicillin-streptomycin at 37 ºC in a humidified atmosphere equilibrated with 5 % CO_2. The CATH.a cells were incubated for 3-8 d. The differentiated CATH.a cells were obtained by incubation for 2-3 d with *N* 6,2'-*O*-dibutyryladenosine 3',5'-monophosphate (dbcAMP 1 mmol/L, Sigma) as a revulsant from d 1. This culture medium all was replaced by cell bath solution at experiment day. The medium used for undifferentiated CATH.a cell culture was without any revulsant or inducement.

Receptor-radioligand binding assay The AT_1 and AT_2 receptors binding with their ligands respectively were detected and determined by radioligand, ^{125}I labeled [Sar^1IIe^8] angiotensin II, and their antagonists, losartan (1 μmol/L) and PD123319 (PD, 1 μmol/L). The specificity of ^{125}I labeled [Sar^1IIe^8] angiotensin II for its receptors was confirmed with hot Ang II and cold one. The total assay volume of 400 μL contained 64 μL 8 % BSA in PBS, 0.4 μL hot Ang II, competitors or antagonists (cold Ang II/ Los/ PD/ Los+PD) 1 μmol/L. Neurocytes 3×10^6 were contained in the total assay volume of 400 μL. The cells were incubated at 4 ºC in refrigerator for over night. After being washed with cold PBS for 4 times, the cells were laid in NaOH 0.1 mol/L at room temperature for 2 h. The ratioactivity of the cell collections was counted with a Backman 5500 γ-counter.

Potassium current recording Delayed rectifier K^+ current (I_{Kv}) was recorded by using the whole cell patch-clamp configuration in voltage clamp mode on CATH.a cells bathed in a solution containing (in mmol/L) NaCl 137, KCl 5.4, $CaCl_2$ 1.35, $MgSO_4$ 2, NaH_2PO_4 0.3, $CdCl_2$ 0.3, dextrose 10, HEPES 10, and TTX 0.0015, pH 7.4 equilibrated with NaOH. The patch pipette microelectrode with resistances of 4-10 MΩ was filled with an internal pipette solution that corresponds with the intracellular fluid containing (in mmol/L) KCl 130, $CaCl_2$ 0.25, $MgCl_2$ 2, ATP 1.0, GTP 0.1, EGTA 5, dextrose 8 and HEPES 10, pH 7.2 equilibrated with KOH. Cell capacitance was cancelled electronically and the series resistance was compensated by 75 %-80 %. All experiments were performed at room temperature (23-24 ºC) with an Axopatch 200B amplifier and a Digit data 1200B interface (Axon Instruments, Burlingame, CA). Currents were mensurated and analyzed by pCLAMP 7.0 software system. Standard recording conditions for K^+ current were achieved by stepping from a holding potential of -40 to +10 mV for 100 ms. Mean currents were measured at 50 ms after the initiation of the test pulse. Current density was calculated by dividing transmembrane current (pA) and membrane capacitance (pF).

Chronotropic firing spark recording Sponta-

neous or elicited action potentials (APs) were observed and recorded using whole-cell patch clamp in current clamp mode in CATH.a cells. The cellular firing rate was measured as the number of fully developed APs that were depolarization potentials beyond 0 mV per second (Hz).

Reagents DMEM was obtained from GIBGO (Grand Island, NY). Crystallized trypsin (1×) was from Cooper Biomedical (Malvern, PA). TTX was purchased from Calbiochem (La Jolla, CA). KN-93, U73122, and Calphostin C (Cal) were purchased from BIOMOL Research Laboratories Inc (Plymouth Meeting, PA). Losartan potassium (Los) was generously provided by Merck (Rahway, NJ). Poly-*L*-lysine (M_r 150 000), Ang II, PD123319, ATP, GTP, $CdCl_2$ and HEPES were purchased from Sigma (St. Louis, USA).

Data analysis Results are expressed as mean± SEM. Statistical significance was evaluated by a one-way ANOVA followed by a Newman-Keuls test. Differences were considered significant at $P<0.05$.

RESULTS

Receptor-radioligand binding test The binding of angiotensin receptor specific radioligand ^{125}I labeled [Sar^{1}IIe^8] Ang II ([^{125}I]-Ang II), an agonist of Ang II receptors, was specific and the number of the total Ang II receptor proteins was (98±10) counts/mg protein (an average value of 3 trails) in CATH.a cells. The binding of a specific ligand of AT_1 receptor, losartan (1 μmol/L) was 65 %±9 % of the total binding of [^{125}I]-Ang II, but for an AT_2 receptor specific ligand, PD123319 (1 μmol/L), it was 34 %±7 % in undifferentiated CATH.a cells. The similar results were also obtained from the differentiated CATH.a cells. Numbers of the total and AT_1 and AT_2 receptors in each microgram protein were 86 %± 9 %, 56 %±4 %, and 27 %±5 %, respectively. These findings, together with the results of RT-PCR previously, suggest that both the AT_1 and AT_2 receptors exist in CATH.a cells, and the AT_1 receptor was expressed predominantly compared with AT_2 receptor ($P<0.01$, $n=9$).

Effect of Ang II on I_{Kv} in CATH.a cells Superfusion of CATH.a cells only with PD123319 1 μmol/L, an AT_2 receptor antagonist, did not affect the I_{Kv}, while superfusion with Ang II 100 nmol/L caused a significant reduction in I_{Kv} from (20.7±1.5) to (13.2±2.4) pA/pF ($P<0.05$, $n=12$) in the presence of PD123319 1 μmol/L. The effect of Ang II was completely inhibited by addition of AT_1 receptor antagonist losartan 1 μmol/L [(19.4±1.8) *vs* (13.2±2.4) pA/pF] ($P<0.05$, $n=12$, Fig 1).

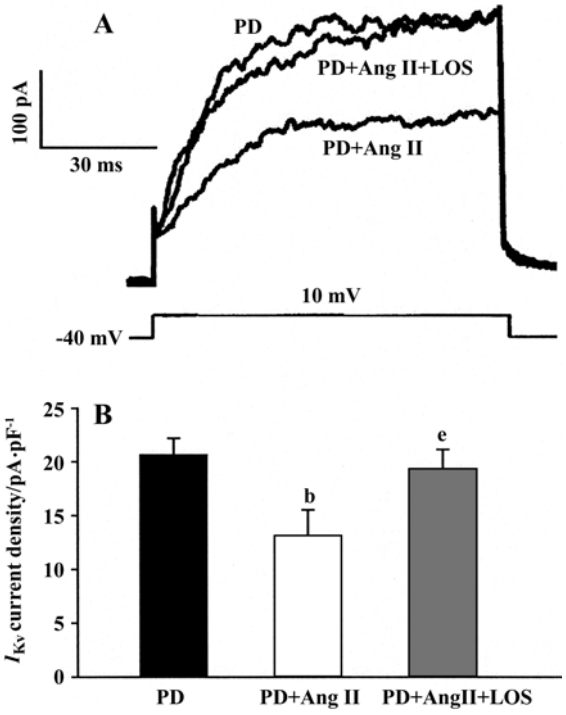

Fig 1. Effect of Ang II 100 nmol/L on I_{Kv} in CATH.a cells in the presence of PD123319 (PD) 1 μmol/L and losartan (LOS) 1 μmol/L. The I_{Kv} was recorded during 100-ms voltage steps from a holding potential -40 mV to +10 mV. A: representative current tracings. B: bar graphs showing I_{Kv} densities. $n=12$. Mean±SEM. $^bP<0.05$ *vs* PD; $^eP<0.05$ *vs* PD+Ang II.

Action of phospholipase C in Ang II-induced inhibition of I_{Kv} in CATH.a cells Superfusion with Ang II (100 nmol/L) decreased the I_{Kv} from (20.1±2.0) pA/pF of the control value to (12.4±1.5) pA/pF ($P<0.05$, $n=8$) in the presence PD123319 1 μmol/L in the bath solution. After washout of Ang II and PD123319 for 10 to 15 min, the I_{Kv} returned to primary levels, and then superfusion of PD123319 plus U73122 (10 μmol/L), an inhibitor of phospholipase C (PLC), did not alter the I_{Kv} [(21±3) pA/pF]. Subsequent superfusion of Ang II (100 nmol/L) in the presence of both PD123319 and U73122 had no effect on the I_{Kv} (20.1±2.8) pA/pF. This result strongly indicates that the PLC is involved in the AT_1 receptor-mediated reduction of I_{Kv} in CATH.a cells (Fig 2).

Role of PKC and CaMK II in Ang II-induced reduction of I_{Kv} In the presence of PD123319 1 μmol/L, Ang II 100 nmol/L greatly reduced the I_{Kv} from (20.1±0.8) to (12.8±0.9) pA/pF ($P<0.01$, $n=10$), and it

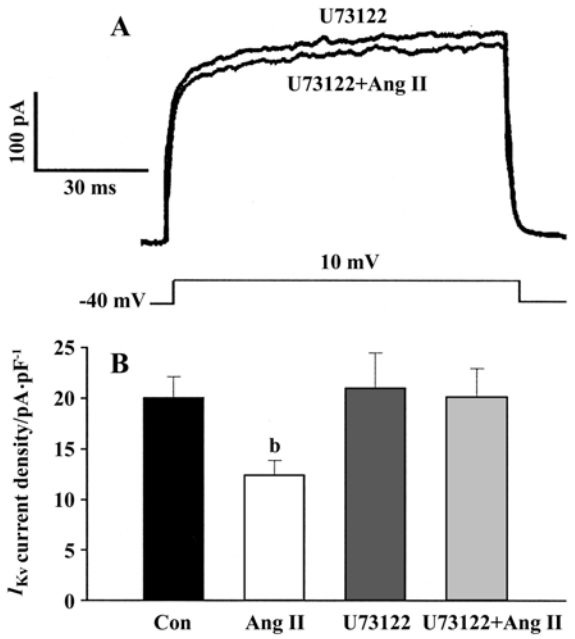

Fig 2. Effect of PLC inhibition on the reduction of I_{Kv} in CATH.a cells induced by Ang II 100 nmol/L in the presence of PD123319 1 μmol/L to block AT_2 receptors. Kv current (I_{Kv}) was recorded during 100 ms voltage steps from a holding potential of -40 mV to +10 mV. A: representative current tracings. B: bar graphs. *n*=8. Mean±SEM. $^bP<0.05$ *vs* control. The concentration of U73122 was 10 μmol/L.

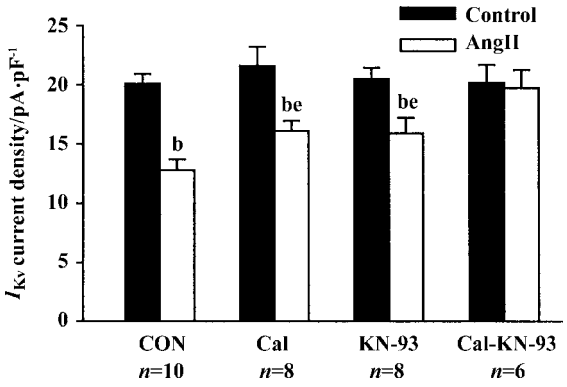

Fig 3. Effects of calphostin C (Cal, 10 μmol/L) and KN-93 (10 μmol/L) on the I_{Kv} reduction induced by Ang II 100 nmol/L in CATH.a cells. The Kv current (I_{Kv}) was recorded during 100 ms voltage steps from a holding potential of –40 mV to +10 mV. All recordings were made under the presence of PD123319 1 μmol/L to block AT_2 receptors. Mean±SEM. $^bP<0.05$ *vs* corresponding control. $^cP<0.05$ *vs* Ang II alone.

was reduced from 21.6±1.6 to 16.1±0.9 ($P<0.05$, $n=8$) by PKC inhibitor calphostin C (Cal, 10 μmol/L) inhibited the reduction of I_{Kv} induced by Ang II [(16.1±0.9) *vs* (12.8±0.9)] . In another group of cells the reduction of I_{Kv} elicited by Ang II via AT_1 receptor also was attenuated from [(15.9±1.3) *vs* (12.8±0.9)] by the selective CaMK II inhibitor KN-93 (10 μmol/L) ($P<0.05$, $n=8$). In contrast, superfusion of KN-92 (10 μmol/L), an inactive analog of KN-93, had no effect on the Ang II-induced inhibition of the I_{Kv} (data not shown). Cal, KN-93, or KN-92 alone had no effect on I_{Kv} (data not shown). However, the inhibition of both PKC and CaMK II by combined superfusion of Cal and KN-93 could completely abolish the reduction of I_{Kv} elicited by Ang II in CATH.a cells. The I_{Kv} was close to the control value [(20.2±1.5) *vs* (19.7±1.5), $P>0.05$, $n=6$). These results indicate that activation of both CaMK II and PKC is required for the Ang II-induced reduction of I_{Kv} (Fig 3).

Chronotropic effect of Ang II on firing rate in CATH.a cells Spontaneous action potentials were observed and recorded using whole-cell patch clamp in current clamp mode in differentiated CATH.a cells. The mean amplitude of those action potentials was (65±4) mV, and the mean resting membrane potential was (-52±5) mV. Superfusion of Ang II (100 nmol/L) and PD123319 (1 μmol/L) greatly increased the firing rate from (0.11±0.04) Hz to (0.44±0.03) Hz ($P<0.01$, $n=5$) in the CATH.a cells (Fig 4). This effect of Ang II was inhibited by the selective AT_1 receptor ligand losartan (1 μmol/L), with the firing rate decreasing to (0.13±0.03) Hz. Losartan alone did not alter firing rate of the cells. These results are consistent with those observed in primary cultured neurons from rat brain[13].

DISCUSSION

Previous studies in neuron cultures have indicated that the angiotensin II (Ang II) is an essential neuropeptide in central control of blood pressure, which activates the neuronal AT_1 receptors and noradrenergic pathways in brain[14]. The increases of calcium current (I_{Ca}) and the decreases of delayed rectifier potassium current (I_{Kv}) and A-type potassium currents induced by Ang II result in an increase of neuronal firing rate[12]. However, due to the heterogeneity of the primary neuronal cultures used in previous studies, the further research of the intracellular signaling transduction involved in the action of Ang II became a quite difficult issue. For this reason, the present study, using the CATH.a catecholaminergic cells derived from the mouse locus cerulean tumor, as a suitable model, investigated the intracellular mechanism of the action of the Ang II on neurons. The results indicated that both the AT_1 and

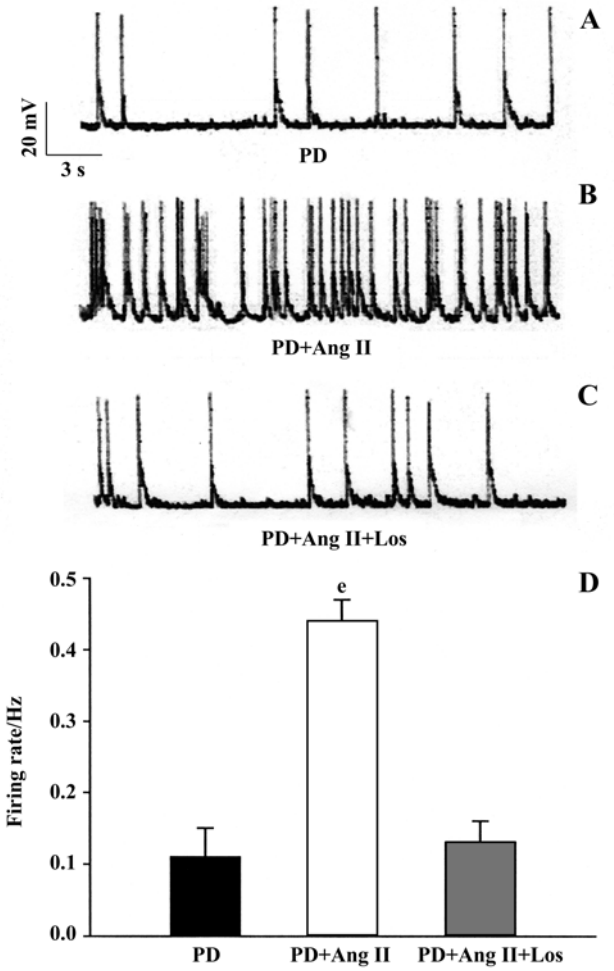

Fig 4. Effect of Ang II on firing rate in differentiated CATH.a cells. A-C: recordings of action potentials from a representative cell under various conditions. A: superfusion of the AT_2 receptor antagonist PD123319 (PD, 1 μmol/L) alone; B: superfusion of Ang II (100 nmol/L) in presence of PD123319 (1 μmol/L); C: superfusion of Ang II (100 nmol/L) plus the AT_1 receptor antagonist losartan (Los, 1 μmol/L) in presence of PD123319 (1 μmol/L); D: bar graphs showing the chronotropic effects of differentiated CATH.a cells with various treatments. *n*=5. Mean±SEM. $^eP<0.01$ *vs* PD alone.

AT_2 receptor were expressed on either the undifferentiated or differentiated CATH.a cells and that the expression of AT_1 receptor was significantly more than that of AT_2 receptor in either undifferentiated or differentiated CATH.a cell. Further results showed that the ability of the undifferentiated CATH.a cells for generating discharges or ionic currents was almost failing which was consistent to a recent study[15] that the CATH.a cells failed to accumulate the noradrenaline, suggesting that the undifferentiated CATH.a cells may lack of a functional NE transporter, while the response of differentiated CATH.a cells with the dibutyryl cyclic AMP was significantly increased, suggesting that the differentiated CATH.a cell may be used as a model for investigation of the modulation mechanisms of catecholaminergic system mediated by the Ang II receptor.

Superfusion of CATH.a cells only with PD123319, an AT_2 receptor antagonist, did not affect the I_{Kv} in CATH.a cells, while Ang II plus PD123319 greatly reduced the Kv current (I_{Kv}), and this effect was completely blocked by addition of losartan (Los), an AT_1 receptor antagonist. These results suggest that the reduction of I_{Kv} induced by Ang II is mediated by the AT_1 receptor, but not by AT_2 receptor. It is assumed that the actions of the Ang II on central catecholaminergic neurons may depend on the mediation of AT_1 receptors which contribute to the cellular membrane depolarization and the firing of catecholaminergic neurons through increase of the norepinephrine release.

Results of this study further revealed that when the PKC antagonist Cal or the CaMK II antagonist KN-93 was alone superfused, the inhibitory effect of Ang II on I_{Kv} was markedly attenuated, suggesting that both the PKC and the CaMK II were involved in mediating the inhibition of Ang II on I_{Kv} in CATH.a cells. In addition, U73122 that inhibits the activation of PLC induced by Ang II can abolish the inhibition of Ang II on I_{Kv} in CATH.a cells. It is possible that the PLC may be a key substance for AT_1 receptor-mediated signal transduction in noradrenergic neurons. Both PKC and CaMK II may play an important role in the different subdivided intracellular signal transduction which mediated the actions of Ang II on the central catecholaminergic neurons.

There is evidence showing that phosphorylation and/or dephosphorylation of Kv channel protein plays an important role in regulation of neuronal activities[16], and that the AT_1 receptor-mediated inhibition of Kv currents results from inhibition of the Kv2.2[17]. Furthermore, the multiple consensus PKC (S/T-X-R/K) and CaMK II (R-X-X-ST) phosphorylation sites are present within the cytoplasmic domains by inspection of the amino acid sequence of the rat Kv2.2 channel protein[17]. Results of the present study indicated that activation of the PLC, PKC and CaMK II delivered by AT_1 receptor may be the molecular mechanism which lead to inhibition of Kv currents. However, it is unclear whether the inhibitory action of AT_1 receptor on the Kv currents is due to a result of the Kv2.2 inhibition, and that whether the exact phosphorylation site of the Kv channel protein is the same as that in neurons. It is required for

further investigation to clarify this issue.

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