Mechanisms of extracellular NO and Ca^{2+} regulating the growth of wheat seedling roots

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Abstract: Our previous studies suggested that crosstalk of nitric oxide (NO) with Ca^{2+} in regulating stomatal movement. However, its mechanisms of action is not well defined in plant roots. Here, sodium nitroprusside (SNP, a NO donor) showed inhibitory effects on the growth of wheat seedling roots at concentration of 10, 50 or 100 μmol L^{-1} respectively,which was alleviated through reducing extracellular Ca^{2+}concentration. Analysising the content of Ca^{2+} and K^+ in wheat seedling roots shows that SNP significantly promotes Ca^{2+} accumulation and inhibites K^+ accumulation at higher concentration of Ca^{2+}, but 10 μmol L^{-1} SNP promotes K^+ accumulation in the absence of extracellular Ca^{2+}. To gain further insights into Ca^{2+} function in NO-regulated the growth of wheat seedling roots, we patch-clamped protoplasts of wheat seedling roots in a whole-cell configuration. In the absence of extracellular Ca^{2+}, NO activates inward rectifying K^+ channels, but has little effects on outward rectifying K^+ channels. Adding 2 mmol L^{-1} $CaCl_2$ to the bath solution, NO significantly activates outward rectifying K^+ channels, which was partially alleviated by $LaCl_3$ (a Ca^{2+} channel inhibitor). In contrast, 2 mmol L^{-1} $CaCl_2$ alone has little effects on inward or outward rectifying K^+ channels. Thus, NO inhibits the growth of wheat seedling roots likely by promoting extracellular Ca^{2+} influx excessively. The increase in cytosolic Ca^{2+} appears to inhibit K^+ influx, promote K^+ outflux across plasma membrane, and finally reduces the content of K^+ in root cells. [Life Science Journal 2010;7(2):80-85]. (ISSN: 1097-8135).

Key words: wheat seedling; Nitric oxide; Calcium; Plasma membrane K^+ channels

1 Introduction

NO is a highly diffusible gas and a ubiquitous bioactive molecule with well-characterized signaling roles in mammalian systems[1]. NO is suggested to play crucial roles in plant development, stress responses and programmed cell death, but its site of action in any signaling pathway remains unknown[2-4]. In organ development of plant roots, NO can replace the role of auxin, by activating its downstream MAPK system to mediate growth and development of lateral and adventitious root [5-7]. In addition, NO increases the main root length in tomato and corn [6,7]. However, the mechanism of NO regulating the growth and development of plant roots was still largely unknwn.

Ca^{2+} is involved in absisic acid (ABA)- and hydrogen peroxide (H_2O_2)-induced stomatal closure as a versatile intracellular messenger [8,9]. Diverse biotic and abiotic stresses elicit a transient increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) [10,11], and plants percept and decode these changes in $[Ca^{2+}]_{cyt}$ leading to specially physiological events [12]. Previous studies have shown that Ca^{2+} regulated the development of plant roots, such as the activity of plasma membrane Ca^{2+}-channel and the elevation of $[Ca^{2+}]_{cyt}$ are necessary for root growth and root hair formation[13,14]. Root absorption of Ca^{2+} primarily through the root elongation zone, and by the plasma membrane hyperpolarization-activated cation channel regulation [15,16]. In *Arabidopsis thaliana*, increasing evidence have illustrated that $[Ca^{2+}]_{cyt}$ oscillations are synchronized to $[Ca^{2+}]_{ext}$ oscillations largely through the Ca^{2+}-sensing receptor CAS, and CAS regulates concentrations of inositol 1,4,5-trisphosphate (IP3), which evokes release of Ca^{2+} from internal stores [17]. This finding is distinct from McAinsh group's report that extracellular Ca^{2+}-induced the elevation of $[Ca^{2+}]_{cyt}$ depend on Ca^{2+} influx[18]. However, the mechanism of extracellular Ca^{2+} regulation growth and development of plant root is not clear.

Previous researches have suggested that NO plays an important role in controling Ca^{2+} channel activity and monitoring the balance of intracellular Ca^{2+} in animal cells[19]. The crosstalk NO with Ca^{2+} forms an intricate networks and participates in the regulation of a variety of physiological processes [19,20]. In the plant defense response, cGMP and cADPR are involved in the NO-mediated signaling pathway[21], but cGMP and cADPR are important member that trigger the initiation of intracellular Ca^{2+} signaling pathways [22]. Our researches have found that crosstalk of NO with Ca^{2+} in regulating stomatal movement [23]. However, whether NO regulates the growth of root by regulating intracellular Ca^{2+} balance or not? Garcia-Mata et al. [3] reported that NO regulated inward rectifying K^+ channel of plasma membrane by the release of Ca^{2+} from intracellular Ca^{2+} stores in *Vicia* guard cells. However, this finding is distinct from Sokolovski's reported that NO efficiently inhibited outward rectifying K^+ channel of plasma membrane, which dependent on the extracellular Ca^{2+} influx[24]. Recently, we found that NO can effectively activate the epidermal cells of wheat root plasma membrane K^+ channels to promote root cell of K^+ absorption[25]. Nevertheless, Whether extracellular Ca^{2+} is involved in the regulations of NO on plasma membrane K^+ channels remain poorly understood in plant cells. To clarify the mechanism of NO and Ca^{2+} in the regulation of root growth, here, we investigated the regulation of extracellular NO and Ca^{2+} on root growth, plasma membrane K^+ channels and the accumulation of cytosolic Ca^{2+} or K^+ in wheat seedling root.

2 Materials and methods

2.1 Plant materials

Seeds of wheat (Triticum aestivum L) for "yumai 49" were used in this study. For seed germination, all seeds were sterilized with 0.1% $HgCl_2$ and sown on 0.6% agar-containing MS medium, then kept for 3 d at 4℃ in the dark to break dormancy. The plates were then transferred to a culture room at 22°C and with a 16-h-light/8-h-dark photoperiod. For seedling growth, 3-d-old seedlings from the germination medium were transferred to sterile culture bottles containing 0.8% agar and 1/2MS medium supplemented with various SNP or Ca^{2+} concentrations as indicated. For morphological examination, the culture bottles were incubated for 8d, at a day/night cycle of 12 h/12 h (0.20 to 0.30 mmol m^{-2} s^{-1}) and the temperature was kept at 22±2℃ for day and 18±2℃ for night respectively.

2.2 Measuring the length and number of wheat seedling roots

Measuring the length and number of wheat seedling roots were performed as described by Wen et.al.[25] with slight modifications. Wheat seedling growth for 8 days was wash cleaned and natural straightened to measure the length of the longest root of wheat seedling, then record the numbers of wheat seedling roots. Each value is the mean of 30 measurements ± standard error (n = 5).

2.3 K^+ and Ca^{2+} Determination

The technique has been described previously[28]. The roots of wheat seedling were rinsed with deionized water three times and then dried at 80℃ to a constant weight after filtration with Whatman paper. A total of 0.1g dry powder samples were then extracted with 5 mL 4 mol L^{-1} HCl at 37℃ overnight to release the free cations and centrifuged at 10,000g for 10min. The resulting supernatants of the extracts were diluted and K^+ and Ca^{2+} were determined with a Z-8000 atomic absorption/flame spectrophotometer.

2.4 Isolation of root cell protoplasts and whole-cell K^+ current recordings

Protoplasts were prepared from 8- to 10-day-old roots of "yumai49" *(Triticum aestivum)*,as described previously[26]. Roots were briefly washed in deionized water before being removed from the plant. After removing the tips, the cortex was stripped from the stele by hand. The tissue was finely chopped, which was enzymatically digested for 2 hr in a solution contained 0.08% pectolyase (Sigma Chemical), 0.25 % BSA, 0.5 mmol L^{-1} ascorbate，pH 6, and osmolality at 650 mOmol Kg^{-1} adjusted with sorbitol, was then filtered using 50-mm nylon mesh and centrifuged at 60*g* for 8 min. The protoplasts could be maintained in ice-cold solution (10 mmol L^{-1} K-glutamate, 2 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} KOH, 10 mmol L^{-1} Mes, 0.1 mmol L^{-1} $CaCl_2$, pH 6, and osmolality at 700 mOmol Kg^{-1} adjusted with sorbitol) and stored on ice before patching experiments. In addition, we were able to distinguish between protoplasts from cortical cells and those from xylem parenchyma, as described before[26], in any root the number of cortical cells is very much greater than the number in the xylem parenchyma, we were more likely to have been using protoplasts from the cortex.

Whole-cell K^+ current recordings were performed as described by Hamill et.al.[27] with some modifications. The protoplasts were placed in bath solutions containing (except where otherwise mentioned, such as 10, 50 μmol L^{-1} SNP or 50 μmol L^{-1} SNP +1 mmol L^{-1} $LaCl_3$ for treatments respectively)10 mmol L^{-1} K-glutamate, 2 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} KOH, 10 mmol L^{-1} Mes, 0.1 mmol L^{-1} $CaCl_2$, pH 6. In addition, 0.1 mmol L^{-1} $CaCl_2$was abolished from above bath solution for the absence of Ca^{2+}. In both case (0.1 mmol L^{-1} Ca^{2+} or not), the osmolarity was adjusted to 700 mOsmol Kg^{-1} with sorbitol. Pipettes were pulled with a vertical puller (model PC-10; Narishige) modified for two-stage pulls. and fire-polished by a microforge (model MF-90; Narishige) before using. The pipette solution typically contained 100 mmol L^{-1} K-glutamate, 2 mmol L^{-1} $MgCl_2$, 4 mmol L^{-1} KOH, 1.1 mmol L^{-1} MgATP, 0.1 mmol L^{-1} $CaCl_2$, 10 mmol L^{-1} Hepes, pH 7.2, and osmolality at 720 mOsmol Kg^{-1} with sorbitol. Data were acquired 15 min after the formation of the whole-cell configuration. After the whole-cell configuration was obtained, the membrane was clamped to -52 mV (holding potential). Whole-cell currents were measured in response to 3s voltage pulse from -190 to +110 mV in 20-mV steps, using an EPC-9 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany). Whole-cell data were low-pass filtered with a cut-off frequency of 2.9 kHz and analyzed with PULSEFIT 8.7, IGOR 3.0, and ORIGIN 7.0 software.

3 Results

3.1 Effects of extracellular Ca^{2+} and NO on growth and development of wheat seedling roots

As shown in Fig.1, SNP showed inhibitory effects on the growth of wheat seedling roots at concentration of 10, 50 or 100 μmol L^{-1} respectively in the presence of 10 mmolL^{-1} extracellular Ca^{2+}. For example, 50 μmolL^{-1}SNP inhibited the growth of wheat seedling roots by 75.29%, and 100 μmolL^{-1}SNP inhibited by 85.87% (Fig.1B), which was alleviated through reducing extracellular Ca^{2+} concentration. Moreover, 10 μmol L^{-1} SNP promotes the growth of wheat seedling roots in the absence of extracellular Ca^{2+} (Fig.1A and B). Meanwhile, SNP significantly enhanced the inhibition of root growth by adding extracellular Ca^{2+} (Fig.1A and B). Nevertheless, extracellular Ca^{2+} alone had little effects on the growth of wheat seedling roots without SNP (Fig.1A and B). Interestingly, the number of fibrous roots of wheat seedlings was less affected at different treatments (Fig.1 A and C). The results suggested that NO efficiently inhibited the growth of wheat Seedlings root maybe through promoting extracellular Ca^{2+} influx and increasing accumulation of cytosolic Ca^{2+} in root cells.

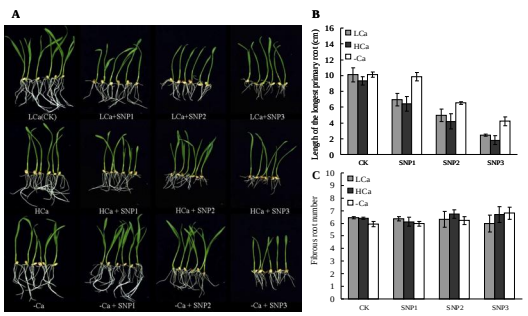

Fig. 1 Regulation of exogenous NO and Ca^{2+} on the growth of wheat seedling roots

A: effects of different treatments on the growth of wheat seedling roots; The concentrations of $CaCl_2$ used are 0 mmol L^{-1} for -Ca, 2 mmol L^{-1} for LCa and 10 mmol L^{-1} for HCa; The concentrations of SNP used are10 μmol L^{-1} for SNP1, 50 μmol L^{-1} for SNP2 and 100 μmol L^{-1} for SNP3; B: effects of exogenous NO and Ca^{2+} on length of primary root; C: effects of exogenous NO and Ca^{2+} on length of fibrous number; Each value in Fig.1-B and C is the mean of measurements with standard error from six independent experiments.

3.2 Effects of extracellular NO on accumulation of cytosolic Ca^{2+} of wheat seedling roots

As shown in Fig.2, increasing concentrations of extracellular Ca^{2+} can elevate the Ca^{2+} content of wheat seedling roots. Interestingly, adding 10 μmol L^{-1} or 50 μmol L^{-1} SNP significantly promote Ca^{2+} accumulation in wheat seedling roots. In the presence of 10 mmol L^{-1} $CaCl_2$, 10 μmol L^{-1}SNP increased Ca^{2+} content by 31.3 %. The results further confirm that NO efficiently inhibited the growth of wheat Seedlings root maybe mainly through modulating extracellular Ca^{2+} influx and increasing accumulation of cytosolic Ca^{2+} in root cells.

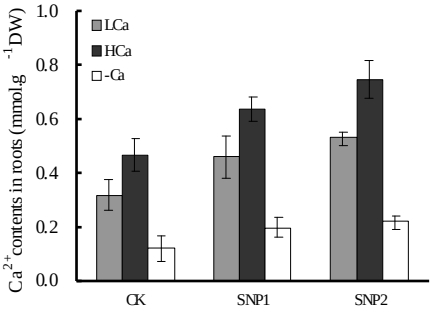

Fig. 2 Effects of exogenous NO on Ca^{2+} content in wheat seedling roots

Abbreviations are the same as in Fig. 1. Each bar represents the mean of measurements with standard error from six independent experiments.

3.3 Effects of extracellular Ca^{2+} and NO on K^+ content of wheat seedling roots

K^+ is the most abundant cation in plant cells and serves as an osmoticum, charge carrier, and enzyme cofactor[29]. Since SNP inhibited the growth of wheat seedlings root in the presence of higer concentration of extracellular Ca^{2+} (Fig.1), we speculate that SNP inhibited the K^+ accumulation in wheat seedling roots. As we speculate, 50 μmolL^{-1}SNP significantly inhibited the K^+ accumulation in wheat seedling roots in the presence of 10 mmolL^{-1} extracellular Ca^{2+} (Fig.3). Interestingly, the removal of Ca^{2+} from the growth medium, 10 mmolL^{-1} SNP or 50μmolL^{-1} SNP promote the K^+ accumulation in wheat seedling roots. However, extracellular Ca^{2+} only had little effects on the K^+ content of wheat seedling root at different concentrations. The results suggested that NO efficiently promoted absorption of Ca^{2+} and increases Ca^{2+} accumulation in wheat seedling root cells. Moreover, a higher $[Ca^{2+}]_{cyt}$ inhibits the absorption of K^+ and affects the growth of wheat seedling roots.

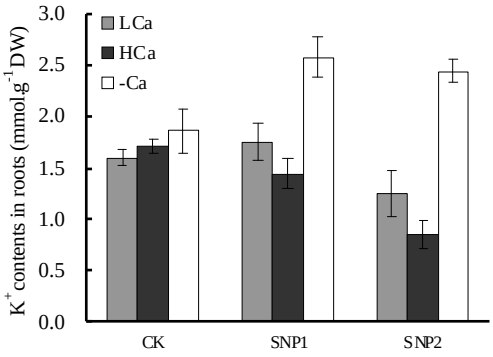

Fig. 3 Effects of exogenous NO and Ca^{2+} on K^+ content in wheat seedling roots

Abbreviations are the same as in Fig. 1. Each bar represents the mean of measurements with standard error from six independent experiments.

3.4 Regulation of extracellular Ca^{2+} or NO on plasma membrane K^+ channels in root cells of wheat seedling

Since the cortical cells of roots are an important component of the route of uptake of nutrients from the soil to the plant and show uptake patterns similar to intact roots[30], we choose the cortical cells for elctrophsiological experiments to understand the mechanism of Ca^{2+}-or NO-regulated K^+ transport across membrane. The results showed that 10 or 50 μmolL^{-1} SNP efficiently inhibited the outward rectifying K^+ channel currents and activated inward rectifying K^+ channel currents in the absence of extracellular Ca^{2+} (Fig.5). It is worthy of noting that 50 μmolL^{-1} SNP significantly activated outward rectifying K^+ channel currents and inhibited the inward rectifying K^+ channel currents when $CaCl_2$ was added to the bath solution, at concentration of 2 mmol L^{-1}.

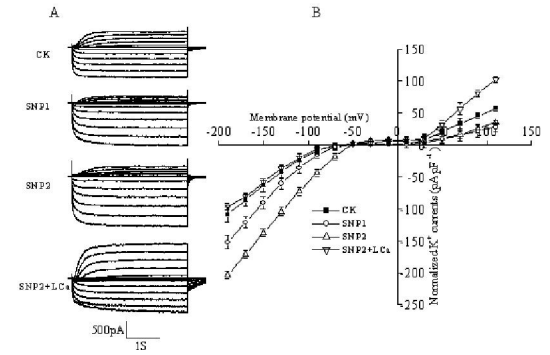

Fig. 4 Regulation of exogenous NO on inward-rectifying and outward-rectifying K^+ channels of the cortical cells in wheat seedling root

A: effects of different treatments on voltage-dependent inward- and outward-rectifying K^+ channels of the cortical cells in wheat seedling root; B: relationship between the whole cell K^+ current (pA) and membrane potential (mV); CK: control; SNP1: 10 μmol L^{-1} SNP; SNP2: 50 μmol L^{-1} SNP; SNP2+LCa: 50μmol L^{-1} SNP + 2mmol L^{-1} $CaCl_2$ treatments. Each value in B is the mean currents from six independent experiments and the error bar denotes the standard error.

Meanwhile, 50 μmolL^{-1} SNP significantly activated outward rectifying K^+ channels and inhibited the inward rectifying K^+ channels in the presence of 2 mmol L^{-1} $CaCl_2$, which was alleviated by La^{3+}, at concentration of 1mmol L^{-1} (Fig.6). In contrast, 2 mmol L^{-1} $CaCl_2$ alone had little effects on inward or outward rectifying K^+ channels (Fig.6). Therefore, we excluded the possibility that effects of extracellular 2 mmol L^{-1} $CaCl_2$ itself on plasma membrane K^+ channels and confirm that extracellular Ca^{2+} was involved in NO-regulated plasma membrane K^+ channels.

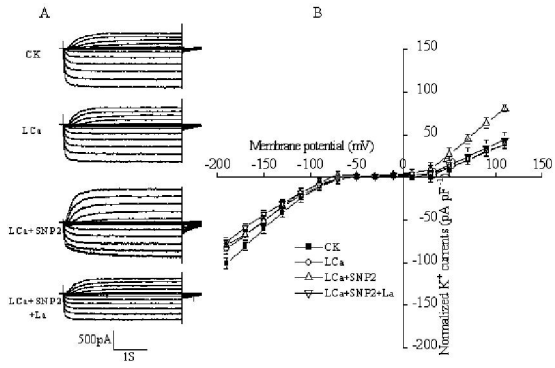

Fig. 5 Crosstalk of NO with Ca^{2+} in regulating K^+ channels of the cortical cells in wheat seedling root

The treatments of A and B are the same as in Fig.4; CK: control; LCa: 2 mmol L^{-1} $CaCl_2$; LCa+SNP2: 2 mmol L^{-1} $CaCl_2$+100 μmol L^{-1} SNP; LCa+SNP2+La: 2 mmol L^{-1} $CaCl_2$+100 μmol L^{-1} SNP+1 mmol L^{-1} $LaCl_3$ treatments. Each value in B is the mean currents from six independent experiments and the error bar denotes the standard error.

4. Discussion

Roots are the primary organs involved in mineral acquisition for plants and function at the interface with the rhizosphere. Although genes whose expression is related to external changes in nutrient composition have been identified, the cascade of cellular responses involved in sensing and signaling nutrient deficiency has not been elucidated[31-33]. NO is suggested to play crucial roles in plant development, stress responses and programmed cell death, but its site of action in any signaling pathway remains unknown[2-4]. In organ development of root, NO can replace the role of auxin, by activating its downstream MAPK system to mediate growth and development of lateral and adventitious root [5-7]. Here, SNP showed inhibitory effects on the growth of wheat seedling roots in the presence of 10 mmolL^{-1} extracellular Ca^{2+} (Fig.1). This finding is distinct from our previous researches that NO increased the length of primary roots and promotes root growth in wheat[25]. However, a key observation favoring the interpretation of this phenomenon is that the inhibitory effects of SNP on the growth of wheat seedling roots was alleviated by reducing extracellular Ca^{2+} concentration. In the absence of extracellular Ca^{2+}, 10 μmol L^{-1} SNP promotes the growth of wheat seedling roots (Fig.1). The results suggested that extracellular Ca^{2+} may be involved in NO-mediated the growth of wheat seedling roots and affect the regulating function of NO on the growth of wheat seedling roots. However, previous studies have shown that Ca^{2+} was involved in the development of plant roots, such as the activity of plasma membrane Ca^{2+}-channel and the elevation of $[Ca^{2+}]_{cyt}$ are necessary for root growth and root hair formation[13,14]. This suggests that in the different conditions, broadly the same pathways are generating the $[Ca^{2+}]_{cyt}$ increase but are possibly activated to different degrees.

Therefore, we offer this hypothesis that NO efficiently inhibited the growth of wheat Seedlings root maybe through promoting extracellular Ca^{2+} influx and increasing excess accumulation of cytosolic Ca^{2+} in root cells. To address this speculation, we therefore investigated effects of NO on accumulation of cytosolic Ca^{2+} of wheat seedling roots. As we expected, SNP obviously promotes Ca^{2+} accumulation in wheat roots at higher concentration of extracellular Ca^{2+} (Fig. 2). In addition, NO as a signal molecule plays an important role in monitoring the balance of intracellular Ca^{2+} in animal cells [20], the crosstalk NO with Ca^{2+} forms an intricate networks and participates in the regulation of a variety of physiological processes [19,20]. Our previous researches have found that crosstalk of NO with Ca^{2+} in regulating stomatal movement [23]. However, it was still unknown about the physiological mechanism of elevated cytosolic Ca^{2+} in regulating the growth and development of plant roots.

K^+ is the most abundant cation in plant cells and serves as an osmoticum, charge carrier, and enzyme cofactor[29]. Since NO efficiently promoted absorption of Ca^{2+} and increases Ca^{2+} accumulation in wheat seedling root cells(Fig.2). Schroeder's group has revealed that the elevated cytosolic Ca^{2+} were well

characterized as potential blockers of K^+ inward rectifying channels and activator of K^+ outward rectifying channels in plant guard cells[35]. we speculate that SNP inhibited the K^+ accumulation in wheat seedling roots under higer concentration of extracellular Ca^{2+}. As we speculate, 10 or 50 $\mu molL^{-1}$SNP significantly inhibited the K^+ accumulation in wheat seedling roots in the presence of 10 $mmolL^{-1}$ extracellular Ca^{2+} (Fig.3). However, extracellular Ca^{2+} only had little effects on the K^+ content of wheat seedling roots. The results suggested that NO efficiently promoted absorption of Ca^{2+} and increases Ca^{2+} accumulation in wheat seedling root cells. Moreover, a higher $[Ca^{2+}]_{cyt}$ inhibits the absorption of K^+ and affects the growth of wheat seedling roots. Interestingly, the removal of Ca^{2+} from the growth medium, 10 $mmolL^{-1}$ SNP promote the K^+ accumulation in wheat seedling roots(Fig.3). Therefore, an alternative explanation is that NO promotes root growth of wheat seedling (Fig.1).

Research has shown that plants absorb K^+ mainly through plasma membrane K^+ channels[26,36]. Recently, we found that NO can effectively activate the epidermal cells of wheat root plasma membrane K^+ channels to promote K^+ absorption and resist drought stress [25]. Since the cortical cells of roots are an important component of the route of uptake of nutrients from the soil to the plant and show uptake patterns similar to intact roots[30], we choose the cortical cells for elctrophsiological experiments to understand the mechanism of K^+ transport across membrane. 10 or 50 $\mu molL^{-1}$ SNP efficiently activated inward rectifying K^+ channel currents and inhibited the outward rectifying K^+ channel currents in the absence of extracellular Ca^{2+}. It is worthy of noting that 50 $\mu molL^{-1}$ SNP significantly activated outward rectifying K^+ channel currents and inhibited the inward rectifying K^+ channel currents when $CaCl_2$ was added to the bath solution, at concentration of 2mmol L^{-1}(Fig. 4). Consistently, the elevated cytosolic Ca^{2+} were well characterized as potential blockers of K^+ inward rectifying channels and activator of K^+ outward rectifying channels in plant cells [35]. Therefore, we offer this hypothesis that extracellular Ca^{2+} regulates K^+ channels entering into root cells. A key observation favoring this hypothesis is that La^{3+} (the special inhibitor of plasma membrane Ca^{2+} channels) significantly alleviated the inhibitory effects of SNP on K^+ channel currents in the presence of 2mmol L^{-1} Ca^{2+} (Fig. 3). An alternative explanation is that plasma membrane Ca^{2+} channels provide a major pathway for extracellular Ca^{2+} entering into root cells. In contrast, 2 mmol L^{-1} $CaCl_2$ alone had little effects on inward or outward rectifying K^+ current. Therefore, we excluded the possibility that effect of extracellular 2 mmol L^{-1} $CaCl_2$ itself on plasma membrane K^+ channel and confirm that extracellular Ca^{2+} was involved in NO-regulated plasma membrane K^+ channels.

5 Conclusion

In conclusion, NO inhibits the growth of wheat seedling roots likely by promoting extracellular Ca^{2+} influx excessively. The increase in cytosolic Ca^{2+} appears to inhibit K^+ influx, promote K^+ outflux across plasma membrane, and finally reducing the K^+ accumulation in wheat seedling roots.

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