LABORATORY INVESTIGATION

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The Effect of Tyrosine Kinase Inhibitors on the L-type Calcium Current in Rat Basilar Smooth Muscle Cells

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Objective : Tyrosine kinase inhibitors may be useful in the management of cerebral vasospasm. It has not yet been reported whether L-type Ca^{2+} channels play a role in tyrosine kinase inhibitors-induced vascular relaxation of cerebral artery. This study was undertaken to clarify the role of L-type Ca^{2+} channels in tyrosine kinase inhibitors-induced vascular relaxation, and to investigate the effect of tyrosine kinase inhibitors on L-type Ca^{2+} channels currents in freshly isolated smooth muscle cells from rat basilar artery.

Methods : The isolation of rat basilar smooth muscle cells was performed by special techniques. The whole cell currents were recorded by whole cell patch clamp technique in freshly isolated smooth muscle cells from rat basilar artery.

Results : Patch clamp studies revealed a whole-cell current which resembles the L-type Ca^{2+} current reported by others. The amplitude of this current was decreased by nimodipine and increased by Bay K 8644. Genistein(n=5), tyrphostin A-23(n=3), A-25(n=6) 30μM reduced the amplitude of the L-type Ca^{2+} channel current in whole cell mode. In contrast, diadzein 30μM (n=3), inactive analogue of genistein, did not decrease the amplitude of the L-type Ca^{2+} channels current.

Conclusion : These results suggest that tyrosine kinase inhibitors such as genistein, tyrphostin A-23, A-25 may relax cerebral vessel through decreasing level of intracellular calcium, $[Ca^{2+}]i$, by inhibition of L-type Ca^{2+} channel.

KEY WORDS : Genistein · Tyrphostin A-23 · Tyrphostin A-25 · Vasospasm · L-type Ca^{2+} channels · Patch-clamp techniques.

Introduction

Vasospasm is the leading cause of disability and death after intracranial aneurysm rupture, but the pathogenesis of the arterial narrowing is not completely understood, and the best form of treatment is not yet clear.

Tyrosine kinases have been shown to be involved in the contraction of peripheral smooth muscle either by activation of receptors or by opening of Ca^{2+} channels[3]. However, little is known of their action in cerebral arteries. Tyrosine kinase may play a role in the regulation of cerebral arterial contraction and tyrosine kinase inhibitors may be useful in the management of vasospasm[11].

Tyrosine kinase may play a role in erythrocyte lysate-induced contraction in rabbit cerebral arteries. However, its action mechanism for cerebral vascular relaxation is not clear. It has not yet been reported whether Ca^{2+} channel plays a role in tyrosine kinase inhibitors-induced vascular relaxation of cerebral artery, although Ca^{2+} channel is important for setting the resting membrane potential and modulating excitability of smooth muscle.

This study was undertaken to clarify the role of Ca^{2+} channel in tyrosine kinase inhibitors-induced vascular relaxation, and to investigate the effect of tyrosine kinase inhibitors on L-type Ca^{2+} currents in freshly isolated smooth muscle cells from rat basilar artery.

The authors investigated the effect of tyrosine kinase inhibitors (30μM)such as genistein, tyrphostin A-23, A-25 on L-type Ca^{2+} channels using patch clamp technique in cerebral smooth muscle cells from rat basilar artery and found that tyrosine kinase inhibitors reduced the Ca^{2+} current.

Materials and Methods

Cell Isolation

The methods for isolation of rat basilar smooth muscle cells have been described[10]. Briefly, Sprague-Dawley male rats were

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anesthetized with Motofane and decapitated. The basilar arteries were removed to a medium consisting of (in mM) : NaCl 130, KCl 5, $CaCl_2$ 0.8, $MgCl_2$ 1.3, glucose 5, N-[2-hydroxyethyl]piperazine-N'[2-ethanesulfonic acid] (Herpes) 10, penicillin (100 units/ml) and streptomycin (0.1g/l).

Arteries were then cleaned of connective tissue and small side branches. The arteries were cut into 2.0-mm rings and incubated for 1 hour at room temperature in a medium containing 0.2mM $CaCl_2$ and collagenase (type II, 0.5g/l), elastase (0.5g/l), hyaluronidase (type IV-S, 0.5g/l) and deoxyribonuclease I (0.1g/l). The rings were washed in fresh solution containing $CaCl_2$ (0.2mM), trypsin inhibitor (0.5g/l) and deoxyribonuclease I (0.1g/l) and then triturated gently. Cells were placed on glass coverslips and stored at 4°C (used in 12 hours) in the abovementioned buffer containing $CaCl_2$ (0.8mM) and bovine serum albumin (2g/l) free of essential fatty acids. Isolated cells stained positive for α-actin and retained the ability to contract in response to KCl, caffeine, serotonin and hemolysate[30].

Whole-Cell Patch Clamp Technique

Cells were voltage-clamped using the whole-cell patch clamp technique[10]. Electrodes were prepared from glass capillary tubing by using a patch electrode puller (KIMAX-51 Kimble products, USA), and pipette resistance was 2~10MΩ. These were positioned using a three-dimensional vernier-type hydraulic micromanipulator (MX-630R, SOMA SCIENTIFIC). Seals (5~10GΩ) were formed by applying gentle negative pressure. Voltage steps were applied with pulse protocols driven by a IBM 586 computer equipped with A-D and D-A converters (DigiData 1200, Axon Instruments Inc., Foster City, Calif., USA). Data of membrane currents were collected and amplified using a patch clamp Axon-patch 1D and pCLAMP 5.7.1 programs (Axon Instruments). None of the record shown were leakage-corrected, and series compensation was not used. Data were filtered with a low-pass Bessel filter (-3 dB at 1 kHz) and digitized on-line at a sampling frequency of 5~10 kHz for subsequent computer analysis. Data analysis was performed using pCLAMP 5/7/1. All experiments were carried out at room temperature (20~26°C).

Membrane seals were made in bath solution containing (in mM) NaCl 125, KCl 5, $MgCl_2$ 1, $BaCl_2$ 10, HEPES 10, glucose 12.5, pH 7.2 with NaOH. For recording of Ca^{2+} channel currents, the bath solution was changed to a solution, which contained (in mM) tetraethylammonium chloride (TEA-Cl) 125, 4-aminopyridine 5, $MgCl_2$ 1, $BaCl_2$ 10 (or $CaCl_2$ 10), HEPES 10, glucose 12.5, pH 7.2 with TEA-OH. The pipette solution contained (in mM) CsCl 135, $MgCl_2$ 4, HEPES 10, Na_2-ATP 2, GTP 0.5,

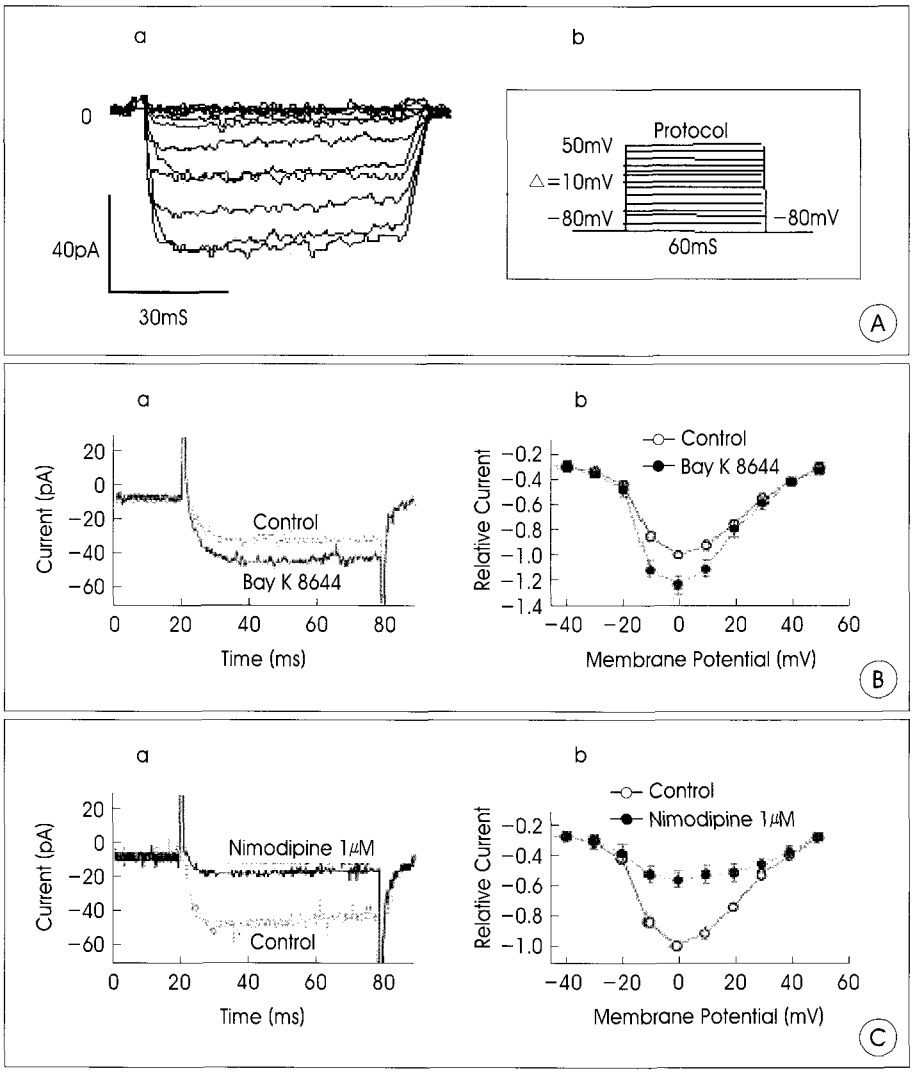

Fig. 1. L-type Ca^{2+} current in isolated smooth muscle cells from rat basilar artery. A : Inward Ca^{2+} current is evoked by depolarization steps from −70 to +50mV in 10mV increments from a holding potential of −80mV. Ba and Ca : The maximal current obtained at 0 mV is plotted to show the effects of 1μM Bay K 8644 (agonist) and 1μM nimodipine (blocker), the dihydropyridines which enhance or block L-type Ca^{2+} current, respectively. Bb and Cb : The current-voltage relationships of the Ca^{2+} current from a holding potential of −80mV in the presence or absence of 1μM Bay K 8644, and 1μM nimodipine.

ethylene glycol-bis (β-aminoethyl-ether) N,N,N',N'-tetraacetic acid (EGTA) 11, $CaCl_2$ 1, pH 7.2 with CsOH [23].

Data analysis

Data are expressed as mean ± SE. Differences among multiple groups were calculated by Student's t-test. A value of $p < 0.05$ was considered statistically significant.

Results

The authors observed that under control conditions the inward current carried by Ba^{2+} remained stable for over 30 min; therefore, all experiments were performed within 3 min. To maximize the Ca^{2+} current, 10mM Ba^{2+} solution was used in the bath instead of the 10mM Ca^{2+} solution, because the Ca^{2+} current has been reported to be inactivated by an increase in $[Ca^{2+}]i$ in some smooth muscle cells. A voltage-dependent Ca^{2+} current was evoked by depolarization potentials from -70 to +50mV in 10mV increments from a holding potential of -80mV. The threshold for activation was around -40mV, and the Ca^{2+} current maximized at around 0mV and reversed at about +50mV (Fig. 1Bb, 1Cb). The Ca^{2+} current was inactivated progressively at more positive potentials but not at potentials near the threshold. The maximal amplitude of the Ca^{2+} current varied within cells studied, from 30 to 200pA, with a mean of 54 ± 6pA (n=12). Reducing the holding potential to -40mV reduced the amplitude of the Ca^{2+} current to almost 50% but did not markedly affect the time course of activation or inactivation of the Ca^{2+} current, indicating the wholecell current was carried by L-type Ca^{2+} channels[16].

Pharmacological studies offered further evidence that this Ca^{2+} current was conducted by L-type Ca^{2+} channels. As shown in an example in figure 1B and 1C, this Ca^{2+} current was blocked by the dihydropyridines nimodipine (1μM; n=4), and was potentiated by Bay K 8644 (1μM; n=8). Examples of the current voltage relationship of this Ca^{2+} current and its sensitivity to Bay K 8644 and nicardipine are as shown (Fig. 1B, C). Bay K 8644, which is thought to activate only L-type Ca^{2+} channels, shifted the

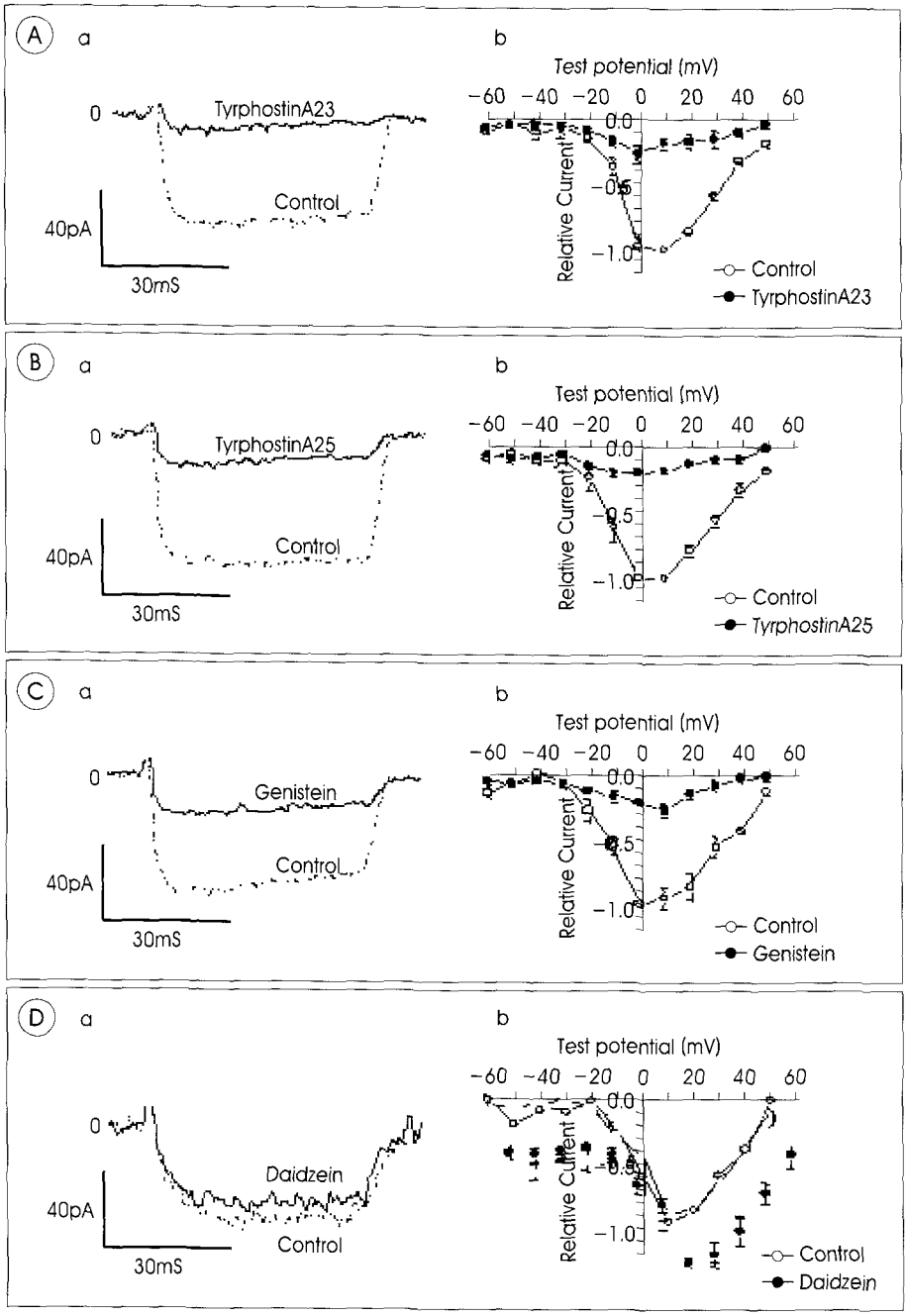

Fig. 2. Effect of tyrosine kinase inhibitors on L-type Ca^{2+} current. Aa : Tyrphostin A23 reduced L-type Ca^{2+} current (obtained at 0mV). Ab. The current-voltage relationship of the study shown in Aa : *p<0.05, Ba, Tyrphostin A23 reduced L-type Ca^{2+} current (obtained at 0mV). B : The current-voltage relationship of the study shown in Ba. *p<0.05, Ca : Genistein reduced L-type Ca^{2+} current (obtained at 0mV). Cb. The current-voltage relationship of the study shown in Ca. *p<0.05, Da : Daidzein didn't reduce L-type Ca^{2+} current. Db. The current-voltage relationship of the study shown in Da. *p>0.05.

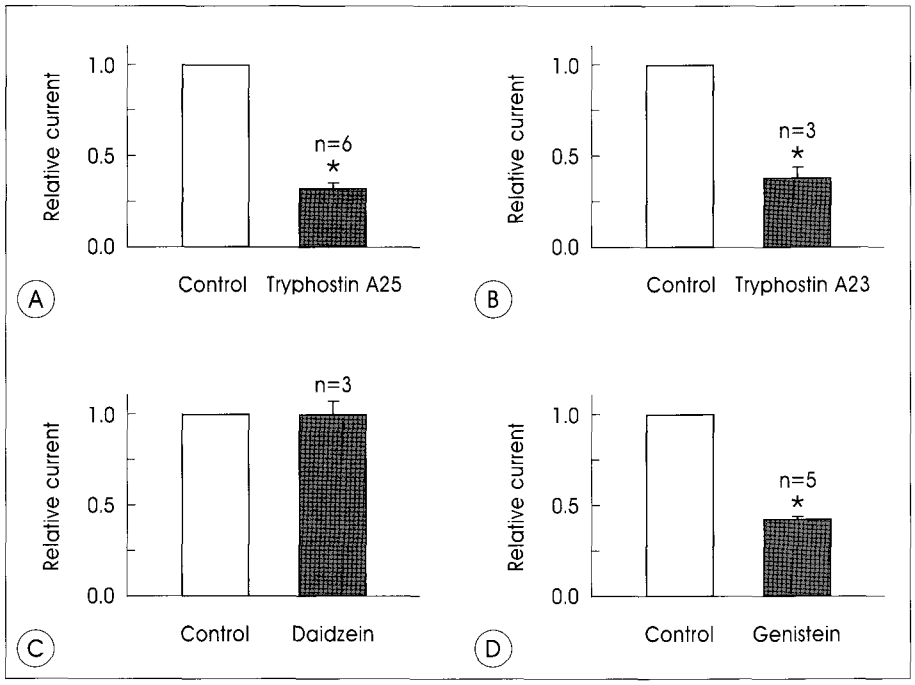

Fig. 3. Summary of the effect of tyrosine kinase inhibitors on L-type Ca^{2+} current induced by a depolarizing pulse of 0mV. *p<0.05.

current voltage relationship to more negative potentials and enhanced the amplitude of the Ca^{2+} current.

The effect of Tyrphostin A23 on L-type Ca^{2+} current was tested by application of 1000μM Tyrphostin A23 (30μL) diluted with normal extracellular buffer, and final concentration of Tyrphostin A23 was 30μM. Administration of Tyrphostin A23 remarkably reduced L-type Ca^{2+} current within 1~3 min (Fig. 2Aa). The current-voltage relationship of the inhibitory effect of Tyrphostin A23 was shown in Fig. 2Ab. Three experiments showed similar effect of Tyrphostin A23 on L-type Ca^{2+} current (Fig. 3B).

The effect of Tyrphostin A25 on L-type Ca^{2+} current was tested by application of 1000μM Tyrphostin A25 (30μL) diluted with normal extracellular buffer, and final concentration of Tyrphostin A25 was 30μM. Administration of Tyrohostin A25 remarkably reduced L-type Ca^{2+} current within 1~3 min (Fig. 2Ba). The current-voltage relationship of the inhibitory effect of Tyrphostin A25 was shown in Fig. 2Bb. Six experiments showed similar effect of Tyrphostin A25 on L-type Ca^{2+} current (Fig. 3A).

Administration of genistein remarkably reduce L-type Ca^{2+} current within 1~3 min (Fig. 2Ca). The current-voltage relationship of the inhibitory effect of genistein was shown in figure 2Cb. Five experiments showed similar effect of genistein on L-type Ca^{2+} current (Fig. 3D).

The effect of Daidzein, inactive analog of genistein on L-type Ca^{2+} current was tested by application of 1000μM Daidzein (30μL) diluted with normal extracellular buffer, and final concentration of Daidzein was 30μM. Administration of Daidzein didn't decrease the amplitude of L-type Ca^{2+} current within 1-3 min (Fig. 2Db). The current voltage relationship of the study was shown in Fig. 2D. Three experiments showed similar effect of Daidzein on L-type Ca^{2+} current (Fig. 3C).

Discussion

It has been established that the etiology of vasospasm is subarachnoid blood clot[2,19,20]. The mechanism for the inhibitory effect of tyrosine kinase inhibitors on erythrocyte lysate-induced contraction is not clear.

Protein tyrosine phosphorylation/dephosphorylation is a key step of signal transduction in cerebral vascular smooth muscle[11]. Action of erythrocyte lysate in cerebral arteries may be associated with an increase in protein tyrosine phosphorylation which participate in the contraction of cerebral smooth muscle.

Tyrosine kinases have been shown to be involved in the contraction of peripheral smooth muscle either by activation of receptors or by opening of Ca^{2+} channels[12]. However, little is known of their action in cerebral arteries.

Tyrosine kinases are key elements in cellular signal transduction pathways and play important roles in the regulation of smooth muscle tone. Tyrosine kinases consist of three general subclasses : (1) the membrane receptors with intrinsic tyrosine kinase domains, such as insulin receptor and receptors for epidermal growth factor and platelet-derived growth factor, (2) membrane-associated non-receptor kinases that are activated by ligand binding and (3) cytosolic non-receptor protein tyrosine kinases, such as the proto-oncogene products Abl and Fes[6]. A large number of potential substrates for these tyrosine kinase, all believed to be directly involved in cell signaling, have been identified, including IP3 receptors[7], phospholipase Cr and MAP kinase[6]. Genistein also inhibits contraction of rat aorta induced by NaF which activates G proteins[1]. In smooth muscle cells, receptor-activation (such as by phenylephrine, 5-HT, vasopressin or endothelin) increases protein tyrosine phosphorylation which regulates both Ca^{2+} release from internal Ca^{2+} stores and Ca^{2+} entry, and produces contraction. Genistein has been reported to inhibit both influx of external Ca^{2+} and release of Ca^{2+} from internal stores, and directly enhance vol-

tage-dependent Ca^{2+} entry in smooth muscle cells from rabbit ear artery[28] and rat myometrial cells[15]. Phosphorylation of tyrosine kinase enhances $[Ca^{2+}]i$ sensitivity of contractile proteins in ileal smooth muscle[25] and potentiates $[Ca^{2+}]i$ independent contraction of rat uterine smooth muscle[4]. Thus, many G protein-coupled receptor agonists, such as angiotensin II, vasopressin, phenylephrine, 5-HT and endothelin, cause $[Ca^{2+}]i$ elevation and smooth muscle contraction mediated at least partially by tyrosine kinase phosphorylation.

Intracellular Ca^{2+}, $[Ca^{2+}]i$, which plays an important role in the regulation of smooth muscle tone, has been shown to increase in major cerebral arteries after subarachnoid hemorrhage (SAH) and has been suggested to mediate the prolonged contraction that is known as vasospasm of those vessels[8,18,19,27]. A massive Ca^{2+} accumulation with formation of intracytoplasmic vacuoles occurred in the smooth muscle cells of canine basilar arteries in vivo 15 min after experimental SAH[13]. Erythrocyte hemolysate, oxyhemoglobin or hemin, have been shown to increase $[Ca^{2+}]i$ in cerebral smooth muscle cells[17,26,30].

Two types of Ca^{2+} channels are mainly involved in mediating Ca^{2+} influx into smooth muscle cells. Ca^{2+} entry can be conducted either through voltage-dependent Ca^{2+} channels that are opened by depolarization or through voltage-independent Ca^{2+} pathways that are opened by receptor activation or emptying of Ca^{2+} stores[22]. Voltage-dependent Ca^{2+} channels have been extensively investigated in smooth muscle cells including those from cerebral vessels[5,9,16,23,24,29] and it has been suggested that L-type Ca^{2+} channel is the predominant channel playing a role in the regulation of cerebral smooth muscle tone.

The Ca^{2+} influx pathways, especially L-type Ca^{2+} channels in cerebral smooth muscle cells, have been investigated using patch clamp techniques. L-type Ca^{2+} have been characterized in rabbit[23], guinea pig[9,24] and rat basilar smooth muscle cells using whole-cell recordings[16,29]. Single-channel study of L-type Ca^{2+} channels in cerebral arteries demonstrated this channel is active in physiological Ca^{2+} concentrations and membrane potentials[5], indicating that L-type Ca^{2+} channels play an active role in the regulation of cerebral smooth muscle tone. A similar Ca^{2+} current which resembles L-type Ca^{2+} current has been reported by others in cerebral smooth muscle cells[9,10,16,23,24,29]. The character of the Ca^{2+} current and the pharmacological response in our studies are consistent with these results.

Since L-type Ca^{2+} channels are predominant and play an important role in initiating contraction in cerebral vascular smooth muscle, L-type Ca^{2+} channel blockers have been used in the prevention and reversal of cerebral vasospasm[13,20]. It is generally believed, however, that L-type Ca^{2+} channel blocking agents possess the ability to improve patient outcome without markedly affecting the diameter of major cerebral vessels[18,21].

Conclusion

The authors can conclude that tyrosine kinase inhibitors may relax cerebral vessel through the decrease of intracellular calcium, $[Ca^{2+}]i$, by inhibition of L-type Ca^{2+} channel.

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Commentary

Calcium influx is generally known to be involved at the cerebral vasospasm after SAH. However, its exact mechanisms still remain unsolved with lots of controversies. Since Ca^{++} entry is crucial for vascular smooth muscle contraction, causing vasospasm, previous studies have addressed the role of tyrosine kinases in the regulation of L-type voltage-gated calcium channels. These channels are also verified well in smooth muscle cell studies using the patch clamp technique (whole cell or cell-attached). The relationship between tyrosine kinases and these channels seems to be complicated, as some studies, which have been performed with cardiac myocytes, provide the conflicting results for their roles[2]. In this study using the whole cell patch clamp technique with myocytes obtained from rat basilar artery, the L-type calcium channel current is consistently demonstrated with the inhibition of tyrosine kinase inhibitors and calcium channel antagonist on this current. Additionally this tendency is also shown in rabbit SAH model where relatively smaller arteries with vasoconstriction are tested for this study[1]. Therefore this study could enhance our knowledge of voltage-gated calcium channels, focusing on the tyrosine kinase inhibitors, while approaching the vasospasm.

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