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Regulation of $Ca_v3.2$ Ca^{2+} Channel Activity by Protein Tyrosine Phosphorylation

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Calcium entry through $Ca_v3.2$ Ca^{2+} channels plays essential roles for various physiological events including thalamic oscillation, muscle contraction, hormone secretion, and sperm acrosomal reaction. In this study, we examined how protein tyrosine phosphatases or protein tyrosine kinases affect $Ca_v3.2$ Ca^{2+} channels reconstituted in *Xenopus* oocytes. We found that $Ca_v3.2$ channel activity was reduced by 25% in response to phenylarsine oxide (tyrosine phosphatase inhibitor), whereas it was augmented by 19% in response to Tyr A47 or herbimycin A (tyrosine kinase inhibitors). However, other biophysical properties of $Ca_v3.2$ currents were not significantly changed by the drugs. These results imply that $Ca_v3.2$ channel activity is capable of being increased by activation of tyrosine phosphatases, but is decreased by activation of tyrosine kinases.

Keywords: $Ca_v3.2$, *Xenopus* oocyte, protein tyrosine kinase inhibitor, protein tyrosine phosphatase inhibitor, voltage clamping

Calcium entry through low-voltage-activated (LVA) Ca^{2+} channels (also called T-type Ca^{2+} channels), of which the activation threshold is around resting membrane potentials, mediates a variety of physiological processes including muscle contraction, low-threshold calcium spikes triggering Na^+-dependent action potentials, acrosome reactions, synaptic plasticity, secretion of hormones, and gene expression [4]. Moreover, these channels have been reported to be coupled with pathophysiological conditions such as absence epilepsy [17], cardiac hypertrophy [22], pain generation [14], and autism spectrum disorders [20].

Protein tyrosine kinases (PTK) are known to be involved in not only cell growth, differentiation [8], apoptosis [2, 23] and sperm motility [10], but also the regulation of various ion channels [1, 15, 24]. However, only a few studies have investigated the regulation of LVA Ca^{2+} channels by protein tyrosine kinases, thereby related information being limited. For example, the inhibition of PTK by Tyr A47 (protein tyrosine kinase inhibitor) increased LVA Ca^{2+} channel activity in mouse spermatogenic cells [5]. On the contrary, application of other PTK inhibitors decreased T-type and N-type current amplitudes in NG108-15 cells [7]. Regulation of LVA Ca^{2+} channels has become much more complicated by the finding that PTK inhibitors such as genistein can inhibit L-type Ca^{2+} and LVA $Ca_v3.1$ channels *via* directly inhibiting the channel rather than affecting tyrosine phosphorylation [3, 11].

In this study, we investigated to examine how $Ca_v3.2$ Ca^{2+} channels reconstituted in the *Xenopus* oocyte system are regulated by PTK. We found that application of phenylarsine oxide (PAO, tyrosine phosphatase inhibitor) significantly reduced $Ca_v3.2$ channel activity. Consistently, application of tyrphostins A47 (Tyr A47, tyrosine kinase inhibitor) increased $Ca_v3.2$ channel activity without affecting other biophysical properties of the $Ca_v3.2$ channels. These findings strongly suggest that $Ca_v3.2$ channel activity can be downregulated by activation of endogenous tyrosine kinases in *Xenopus* oocytes.

$Ca_v3.2$ (α_{1H}; GenBank Accession No. AF051946) cRNA was synthesized using T7 RNA polymerase, and 1 ng of $Ca_v3.2$ cRNA was injected into each oocyte that had been prepared as previously described [6, 13]. Barium inward currents were measured using a two-microelectrode voltage clamp amplifier (OC-725C; Warner Instruments, CT, U.S.A.) from the 3rd day after cRNA injection in the oocytes. Peak currents and exponential fits to currents were analyzed using Clamfit software (Axon Instruments, CA, U.S.A.).

Application of a test potential of −20 mV from a holding potential of −90 mV did not elicit significant currents from the oocytes injected with H_2O or 100 mM KCl (data not shown). These facts suggest that the *Xenopus* oocytes used for this study do not express or barely express endogenous T-type channels. On the contrary, robust inward currents were evoked in response to the same test potential from *Xenopus* oocytes where $Ca_v3.2$ Ca^{2+} channel cRNA was injected, indicating that exogenous $Ca_v3.2$ Ca^{2+} channels were well expressed in the expression system (data not

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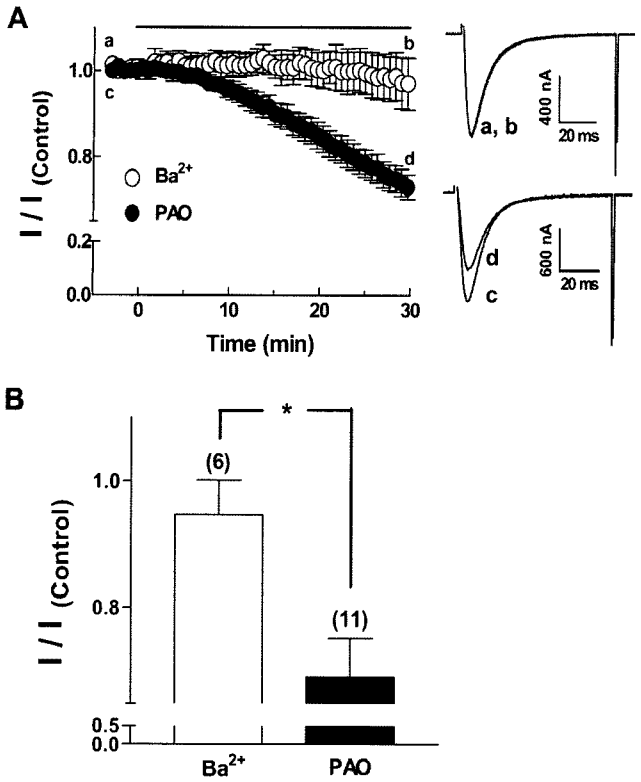

Fig. 1. Effect of PAO on $Ca_v3.2$ channel activity.
A. Time course of PAO-mediated stimulation on $Ca_v3.2$ channel activity. Currents were elicited by a test potential at -20 mV from a holding potential of -90 mV every 15 sec. $Ca_v3.2$ currents were normalized to the current amplitude just before application of 5 μM PAO solution (Sigma-Aldrich, MO, U.S.A.), and the average percentages of current amplitude were plotted. $Ca_v3.2$ current activity was not significantly run-up or run-down during 30 min perfusion of 10 mM Ba^{2+} solution (Control, ○; n=6, a & b), while $Ca_v3.2$ current activity was reduced by application of 5 μM PAO over 30 min (PAO, ●; n=11, c & d). **B.** The normalized current activity of $Ca_v3.2$ was decreased down to 69.2±6.0% (mean±SEM) and the significant differences of the results were evaluated by Student's *t*-test: *, $P<0.05$.

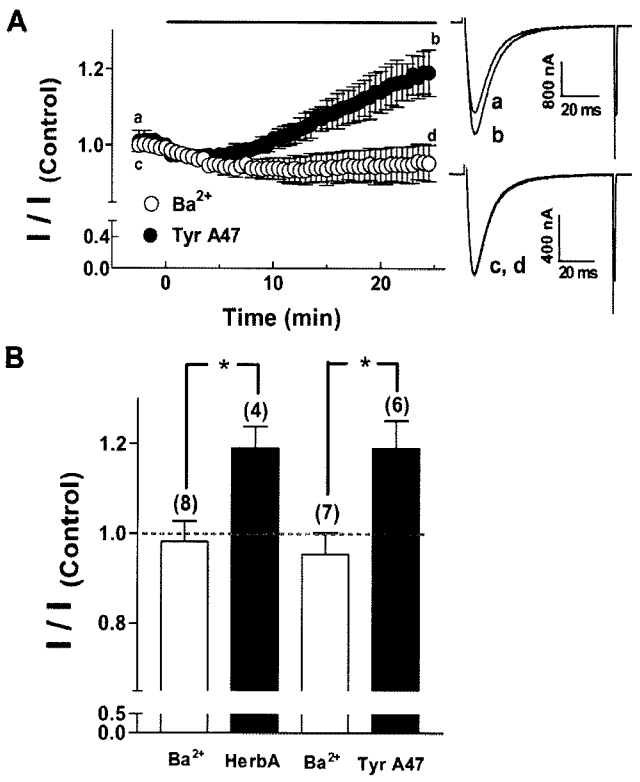

Fig. 2. Effect of Tyr A47 and herbimycin A on $Ca_v3.2$ channel activity.
A. Time course of Tyr A47-mediated stimulation on $Ca_v3.2$ channel activity. $Ca_v3.2$ current activity during 25 min perfusion of 10 mM Ba^{2+} solution showed no significant run-up or run-down (Control, ○; n=7, c & d), while application of 10 μM Tyr A47 for 25 min increased the $Ca_v3.2$ current activity (Tyr A47, ●; n=6, a & b). **B.** The normalized current activity of $Ca_v3.2$ was enhanced up to 19.0±6.0% and 19.0±4.7% after application of 10 μM Tyr A47 and 1 μM herbimycin A, respectively. Significant differences of the results were evaluated by Student's *t*-test: *, $P<0.05$. Tyrphostins A47 and herbimycin A were purchased from Sigma-Aldrich.

shown). Analysis of the recorded currents displayed that these biophysical properties of $Ca_v3.2$ Ca^{2+} channels were very similar to those previously reported [12, 13, 18].

Superfusion of 10 mM Ba^{2+} solution did not significantly change the $Ca_v3.2$ current amplitude in response to the test potential of -20 mV, from a holding potential of -90 mV every 15 sec over 30 min (Fig. 1A, n=6). In contrast, application of 10 mM Ba^{2+} solution containing 5 μM PAO (phenylarsine oxide, of tyrosine phosphatase inhibitor) began to decrease the $Ca_v3.2$ current amplitude after a lagging time of 5–7 min, and the amplitude was diminished by 25.4±3.6% over 30 min (Fig. 1A, n=11). The downregulation effect on $Ca_v3.2$ implies that $Ca_v3.2$ activity was decreased by indirect stimulation of PTK in the oocytes, possibly resulted from inhibition of tyrosine phosphatases by PAO. This PAO effect is consistent with the previous report that the tyrosine phosphatase inhibitor decreased the T-type current amplitude in mouse spermatogenic cells [5].

To further examine the implication, we tested whether $Ca_v3.2$ channel activity can be increased by inhibition of tyrosine kinase(s). When 10 μM Tyr A47 or 1 μM herbimycin A (protein tyrosine kinase inhibitors) was superfused to an oocyte positioned at the chamber for 25 min, the $Ca_v3.2$ current amplitude was slowly increased by 19.3±6.0% or 19.0±4.7%, respectively, with a lagging time of 5–8 min (Fig. 2). Notably, the typical enhancement effects of Tyr A47 or herbimycin A on $Ca_v3.2$ current amplitude were detected from oocytes of 5 batches out of 12, whereas there were no significant changes from the other 7 batches, suggesting that tyrosine phosphorylation effects could be diverse depending on batches of oocytes.

We also examined whether the down- or upregulation effects on $Ca_v3.2$ channel activity by PAO or Tyr A47 were accompanied with modification of other biophysical properties of $Ca_v3.2$ channels. Comparison of current-voltage relationships, steady-state inactivation, and recovery recorded by a series of voltage-protocols before and 25 min after

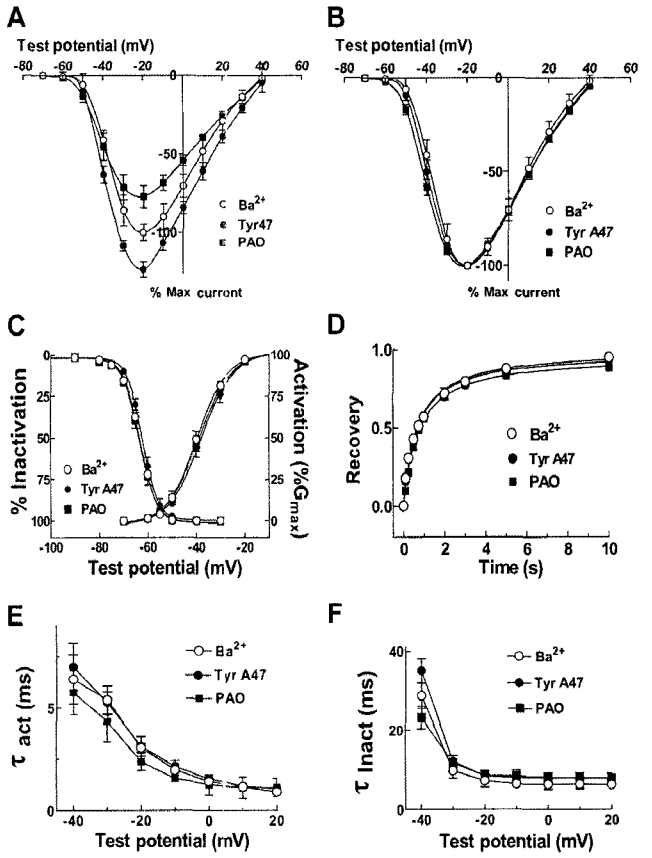

Fig. 3. Biophysical properties of $Ca_v3.2$ before and after treatment with PAO and Tyr A47.

A. Normalized I-V relationships. Voltage steps were depolarized by 10 mV increments from -70 mV to +40 mV from a holding potential of -90 mV. Peak currents obtained during test potentials were normalized to the maximum observed before application of 5 μM PAO or 10 μM Tyr A47. **B.** Peak currents of test potentials were normalized to the maximum peak current (-20 mV) observed in each case (Control, ○; Tyr A47 application, ●; PAO application, ■). **C.** Steady-state inactivation and activation curves of the control (○), 25 min after Tyr A47 application (●), and 30 min after application of 5 μM PAO (■). Steady-state inactivation properties were tested during voltage steps to -20 mV from prepulse potentials varying from -100 mV to -30 mV in 10 mV increments. Activation curves were attained by plotting chord conductances (G), calculated by dividing the current amplitude by driving force (apparent reversal potentials-test potentials), normalized to the peak conductance obtained, averaged, and then plotted against the test potential. **D.** Recovery time courses of the control (○), 25 min after 10 μM Tyr A47 treatment (●), and 30 min after 5 μM PAO treatment (■). Relative peak currents of the test pulse depending on the interpulse duration time were plotted and then fitted by single exponential association. Data represent mean±SEM. **E** and **F**, Average activation (E) and inactivation (F) time constants of $Ca_v3.2$ currents before (○) and after treatment of 10 μM Tyr A47 (●) and 5 μM PAO (■) were plotted as a function of test potentials.

application of PAO or Tyr A47 displayed that, except for the down- or upregulation of $Ca_v3.2$ current amplitude, no significant differences were found before and after those drugs in normalized current-voltage relationships, half-inactivation potentials for steady-state inactivation, half-activation, and recovery time constants (Fig. 3).

The $Ca_v3.2$ Ca^{2+} channel has been identified to be the main LVA Ca^{2+} channel isoform expressed in mammalian spermatogenic cells and involved in the acrosome reaction [9, 16, 19, 21]. LVA Ca^{2+} channel activity in mouse spermatogenic cells was reported to be downregulated by inhibiting the protein tyrosine phosphatases [5]. On the basis of these findings, we investigated whether the inhibition of protein tyrosine phosphatase by phenylarsine oxide (PAO) can similarly modulate $Ca_v3.2$ Ca^{2+} channels reconstituted in the *Xenopus* oocyte expression system. Consistent with the previous report [5], we found that $Ca_v3.2$ channel activity was reduced by the protein tyrosine phosphatase inhibitor, suggesting that the reduction of $Ca_v3.2$ channel activity might be mediated *via* phosphorylation of endogenous protein tyrosine kinases in oocytes, of which the activity is indirectly stimulated with inhibition of the protein tyrosine phosphatases by PAO. This suggestion was supported by the upregulation effect of $Ca_v3.2$ channel activity by Tyr A47 or herbimycin A. Although the $Ca_v3.2$ channel activity was down- and upregulated by the protein tyrosine phosphatase inhibitor and the protein tyrosine kinase inhibitors, respectively, none of the biophysical properties of the $Ca_v3.2$ channel, including half activation and inactivation voltages, activation and inactivation kinetics of currents, and recovery rates, were significantly changed by the drugs. A simple interpretation for the up- or downregulations of $Ca_v3.2$ channel activity is that the channel activity changes might be caused by changes of the numbers or opening probability of the channels in the plasma membrane. Alternatively, it is possible that PAO and Tyr A47 (or herbimycin A) might change the phosphorylation level(s) of unidentified proteins interacting with the $Ca_v3.2$ channels rather than the channels themselves, inducing up- or downregulation of the $Ca_v3.2$ channel activity.

In conclusion, we have reported here that PAO is capable of decreasing the current amplitude of $Ca_v3.2$, whereas Tyr A47 and herbimycin A increase the amplitude. These findings contribute to list up the $Ca_v3.2$ Ca^{2+} channel in the protein group that could be regulated by the activation

Table 1. Summary of the voltage-dependent properties of $Ca_v3.2$ before and after application of PAO or Tyr A47.

Drugs	Activation			Inactivation		
	V_{50} (mV)	k	τ_{act} (ms)	V_{50} (mV)	k	τ_{in} (ms)
Control	-39.60±0.35	6.23±0.34	3.05±0.21	-63.20±0.24	3.40±0.21	7.23±1.56
PAO	-38.72±0.39	6.63±0.38	2.37±0.41	-63.43±0.21	3.52±0.18	8.60±1.22
Tyr A47	-37.93±0.66	6.67±0.64	3.12±0.48	-62.22±0.13	3.18±0.11	8.39±1.45

of protein tyrosine kinases or tyrosine phosphatases, although the detailed mechanism and physiological relevance of the PTK mediated regulatory effects of the $Ca_v3.2$ channel remain to be further explored *in vivo*.

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