Voltage-sensing phosphatase reveals temporal regulation of TRPC3/C6/C7 channels by membrane phosphoinositides

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TRPC3/C6/C7 channels, a subgroup of classical/canonical TRP channels, are activated by diacylglycerol produced via activation of phospholipase C (PLC)-coupled receptors. Recognition of the physiological importance of these channels has been steadily growing, but the mechanism by which they are regulated remains largely unknown. We recently used a membrane-resident *Danio rerio* voltage-sensing phosphatase (DrVSP) to study TRPC3/C6/C7 regulation and found that the channel activity was controlled by PtdIns(4,5)P$_2$-DAG signaling in a self-limiting manner (Imai Y et al. The Journal of Physiology 2012). In this addendum, we present the advantages of using DrVSP as a molecular tool to study PtdIns(4,5)P$_2$ regulation. DrVSP should be readily applicable for studying phosphoinositide metabolism-linked channel regulation as well as lipid dynamics. Furthermore, in comparison to other modes of self-limiting ion channel regulation, the regulation of TRPC3/C6/C7 channels seems highly susceptible to activation signal strength, which could potentially affect both open duration and the time to peak activation and inactivation. Dysfunction of such self-limiting regulation may contribute to the pathology of the cardiovascular system, gastrointestinal tract and brain, as these channels are broadly distributed and affected by numerous neurohormonal agonists.

DrVSP and CiVSP: Time-solved Tool for Channel Regulation

Recognition of the importance of ion channel regulation by phosphoinositides (PIPs), especially phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P$_2$), continues to grow.[1] In earlier studies, the effects of PtdIns(4,5)P$_2$ application were examined directly, or a PtdIns(4,5)P$_2$ antibody, PtdIns(4,5)P$_2$ scavenger (poly-l-lysine) or a pharmacological inhibitor (wortmannin or quercetin) was used to reduce the membrane PtdIns(4,5)P$_2$ content. These methods remain solid and are well established, but their utility is limited to assessing steady-state PtdIns(4,5)P$_2$ levels. By contrast, methods for using a chemically inducible PIP control system and voltage-sensing phosphatase (VSP) are becoming a standardized means of surveying dynamic PIP regulation. Chemical induction of PIPs is well described in a recent review article.[2] In this addendum, therefore, we have focused on the advantages of using VSP to study channel regulation.

So far VSPs from two aquatic species, *Ciona intestinalis* (Ci) and *Danio rerio* (Dr), have been identified. Ci- and DrVSP exhibit only small differences, mainly in their voltage-sensitivities and expression levels, and their catalytic activities (PI5-phosphatase) and substrate phosphoinositide (PI) specificities are nearly identical.[3] Nonetheless, we think that DrVSP may be somewhat better suited for studying PI-mediated regulation of ion channels. This is because gating currents indicate the level of DrVSP expression in HEK cells to be two to three times higher than that of CiVSP, and because the voltage-sensitivity of DrVSP is shifted rightward by about 50 mV ($V_{1/2}$ values from the Q-V curves are 94 and 44 mV for DrVSP and CiVSP, respectively).[4] Considering that
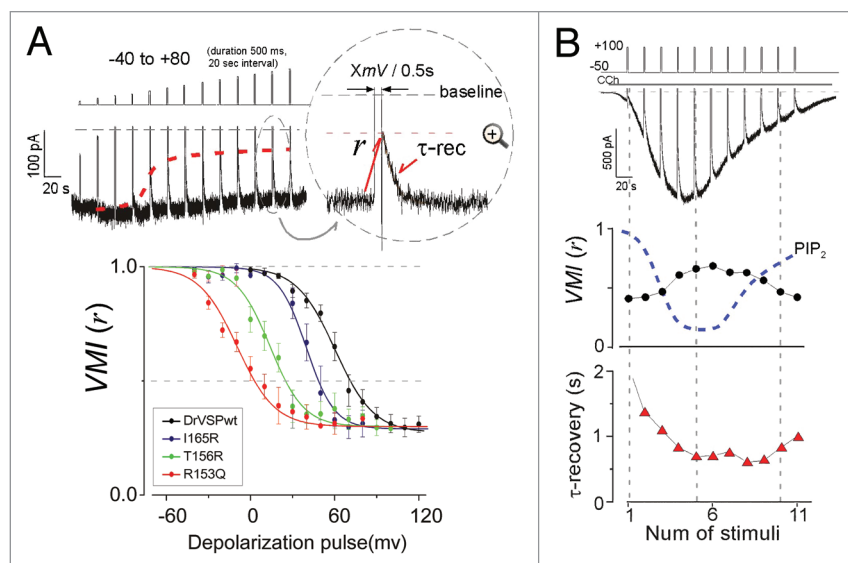
Figure 1. DrVSPs on TRPC currents. (A) Top: Exemplar of the voltage-dependence of VMI (r) of TRPC3/C6/C7 currents observed in HEK cells. TRPC6 currents were evoked by external application of the DAG lipase inhibitor RHC80267 (100 µM). r indicates the residual current after depolarization. The red arrow shows the transient inhibition elicited by the depolarization. Bottom: VMI of TRPC6 currents plotted against depolarization pulse amplitude applied in the presence of the indicated DrVSP mutants (n = more than 4). Note that the Q - V curves of the mutants are also shifted leftward [$V_{1/2}$ (OFF) values for R153Q, T156R and I165R are 16, 73 and 60 mV, respectively].[4] (B) Top: Atypical inhibition trace obtained from HEK cells co-transfected with TRPC6 and wild-type DrVSP. Brief depolarizations (+100 mV, 500 ms) were applied every 10 sec (protocol displayed in top), and currents were evoked by CCh (100 µM). Middle and bottom: r and τ-recovery are plotted against stimulus number from the upper trace. The blue dashed line in the middle part suggests DrVSP-available PtdIns(4,5)P$_2$ (speculative). The typical trace, averaged r and averaged τ-recovery data were shown in reference 11.

the resting membrane potential is around -40 to -10 mV in HEK cells,[5-7] the enzymatic phosphatase activity driven by the resting potential is more negligible with DrVSP. In addition, the rather steep Q - V curve for DrVSP would be expected to yield more dramatic effects. When we combined DrVSP with TRPC channels in HEK cells, a depolarizing pulse to +100 mV for 500 ms was sufficient to produce maximum channel inhibition through depletion of PtdIns(4,5)P$_2$ (referred to as VMI, VSP-mediated inhibition was evaluated based on the residual current (r) before and after the depolarization). However, such robust depolarizations can have unfavorable effects on channel regulation, e.g., the voltage-dependent inhibition relief observed with high voltage-gated Ca channels.[8] To exclude this possibility, we confirmed our results using various voltage-sensing DrVSP derivatives [VMI (r) - V curve], as shown in **Figure 1A**. Note that the V-shifted DrVSPs all inhibited TRPC currents to the same degree

as wild-type DrVSP. These data support our conclusion and further emphasize the advantages of using DrVSP to regulate the activities of a variety of ion channels, including voltage-gated channels.[9,10]

Dynamics of Inhibition and Recovery from the Inhibition by VSP Activation

VSP has enormous potential for use as a molecular tool with which to clarify the PtdIns(4,5)P$_2$ sensitivity and binding kinetics of ion channels, even during periods when ionic currents are flowing. In **Figure 1B,** we present an atypical example of the inhibition of carbachol (CCh)-induced TRPC6 currents (upper) via VSP activation and subsequent current recovery. In contrast to the typical and averaged data presented in our recent study,[11] in 2 of 11 cells tested, we observed responses like those depicted in the figure [i.e., there was a clear bell- or U-shaped relationship between stimulus number and VMI (r)

(middle) and τ-recovery from the inhibition (bottom)]. The bell-shaped r curve was closely related to the peak CCh-induced current. Moreover, τ-recovery accelerated as the CCh-induced current and the value of r became larger. This observation raises the possibility that even during agonist-stimulated macroscopic activity in living cells, VMI magnitude may provide a clue to the level of PtdIns(4,5)P$_2$ binding to the channels, as well as to the kinetics of evoked changes in PtdIns(4,5)P$_2$ binding. The latter would also reflect to some degree PtdIns(4,5) P$_2$ re-synthesis, which would be expected to influence the observed response. In an earlier study, Hardie et al. showed dynamics of living PtdIns(4,5)P$_2$ which was accompanied with the light response in Drosophila photoreceptors by measuring currents through the PtdIns(4,5) P$_2$-sensitive Kir2.1 channel.[12] However, we suggest the use of ectopic VSP is an alternative or a more convenient approach because VSP does not itself produce ionic flow other than gating currents.

Self-limited Ion Channels

Because bioelectric signals are largely attributable to the flow of ions, it is critically important to maintain ion channel activities for appropriate durations. To shorten the duration of ion flow, channels, particularly those contributing to excitation, often possess self-limiting regulatory systems. For instance, **Figure 2A–C** illustrate the mechanisms of self-limiting regulation found in voltage-gated sodium channels, high voltage-gated calcium channels and inotropic ATP receptors. The mechanism underlying the self-limiting regulation of TRPC3/C6/C7 channels (**Fig. 2D**) clearly differs from the other examples shown. TRPC3/C6/C7 channels are intracellular ligand-gated channels assembled as homo- or heterotetramers, and are activated by DAG produced through a reaction catalyzed by G protein- or receptor tyrosine kinase-coupled PLC. But when DAG is produced from its substrate PtdIns(4,5)P$_2$, the resultant reduction in membrane PtdIns(4,5) P$_2$ content independently inhibits channel activation. Thus both activation and inhibition are simultaneously induced by

PLC-catalyzed degradation of PtdIns$(4,5)$P$_2$. As a result, the time required for the response to reach the first current peak is more susceptible to modulation than the other modes of self-limiting regulation.

TRPC channels are often activated by neurohormones released from autonomic nerves and can be thought of as being downstream of the autonomic nervous system (ANS). The ANS is important for maintenance of the stable internal physiological conditions often referred to as "homeostasis". This makes the self-limiting regulation of TRPC channels interesting in part because the resultant regulation of the global effects of the ANS appears to arise from the molecular level.

Channel Regulation Linked to Enzymatic Reaction

Enzymatic reactions are often involved in cell signaling through, for example, an increase in the concentration of a product. On the other hand, the functionality of substrate reduction or depletion is easily masked by the reduction in the productivity of the catalytic reaction. Consequently, signaling systems mediated by enzymatic reactions might be expected to exhibit physiologically bimodal biochemical responses. The self-limiting regulation linked to PLC activity could thus provide profound mechanistic insight of channel modulation as well as rediscovering subtle feature of enzymatic functionality. Furthermore, when this regulation is disrupted which can be seen experimentally in the case of CCh-induced currents in the presence of an excess of the substrate analog dic8-PtdIns$[(4,5)P_2]$, the decay phase of the currents is prolonged (**Fig. 2B**), and the ensuing buildup of cytosolic Na$^+$ and/or Ca^{2+} can have significant pathophysiological effects that could underlie the development of such ailments as vascular hypertension, cardiac hypertrophy and renal failure.

Overall, our results emphasize that TRPC3/C6/C7 channels are subject to self-limiting regulation that is related to their close association with the PtdIns$(4,5)$P$_2$-PLC-DAG cascade and is distinct from the self-limiting mechanisms observed in voltage-gated channels.
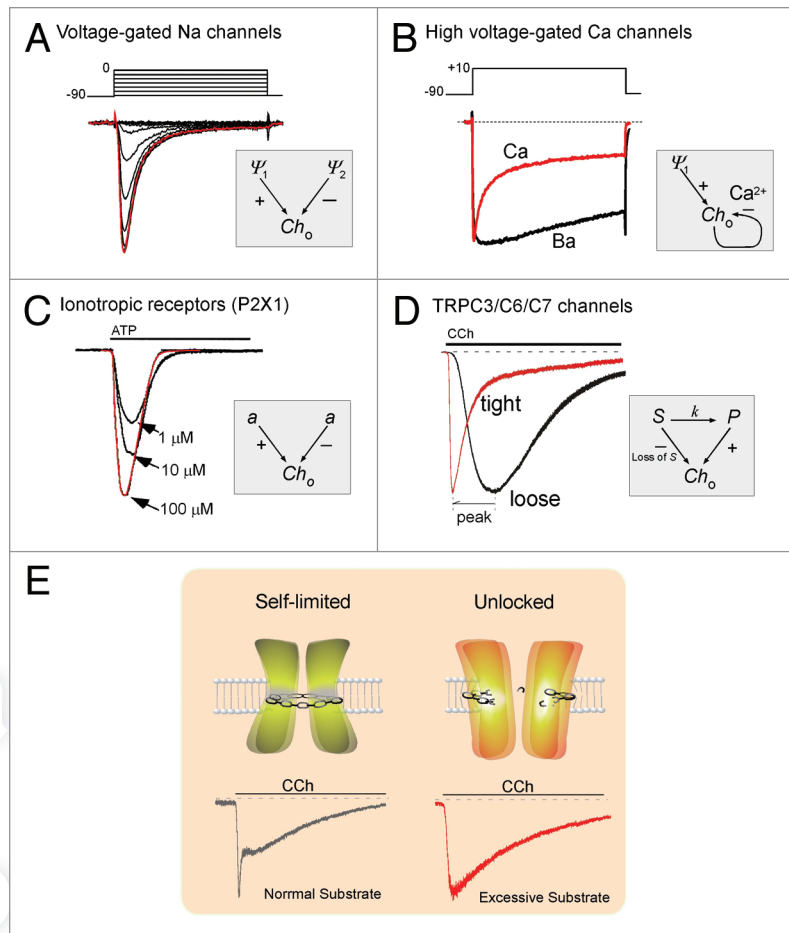

Figure 2. Self-limiting regulatory systems in ion channels. Schemes for channel opening (Ch_o). (A) Voltage-gated sodium channels (rNa$_V$1.2) in vertebrates open (+) and quickly inactivate (-) in response to depolarization (ψ_1 and ψ_2: difference in the potentials coordinate the V-shaped current trace). (B) High voltage-gated Ca channels (Ca$_V$1.2) open upon membrane depolarization (ψ_1) and inactivate due to negative feedback regulation wherein Ca^{2+} permeating through the channels inactivates the channels (calcium-dependent inactivation). (C) Ligand-gated cys-loop receptors (P2X1) are activated and then desensitized by the concentration-dependent binding of an agonist (a). Copyright (1996) National Academy of Sciences, USA. (D) TRPC3/C6/C7 channels are activated by DAG, a product of PtdIns$(4,5)$P$_2$, and the resultant reduction in PtdIns$(4,5)$P$_2$ independently inhibits channel opening. Strength linkages with G protein-coupled receptors alter the kinetics in TRPC6 currents (tight and loose linkages are represented by the red and black traces, respectively). The respective current traces were obtained from reference 11 and 13–15. (E) Currents obtained under self-limiting conditions exhibit faster inactivation than those obtained under unlocked conditions induced by application of excessive PtdIns$(4,5)$P$_2$ through the patch-pipette (TRPC7 currents obtained from ref. 11).

The specific physiological importance of this type of regulation, as compared with other modes of channel regulation, will be an important area of investigation in the future.

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