

## An oily competition: role of $\beta$ subunit palmitoylation for $\text{Ca}^{2+}$ channel modulation by fatty acids

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In electrically excitable cells, voltage-gated calcium channels (VGCCs) serve a critical signaling role by translating changes in membrane potential to calcium ion influx. The resulting intracellular calcium signals are perfectly tuned to cellular needs in terms of amplitude, spatial distribution, and timing. This tuning is accomplished by a structural diversity of these channels and close functional cross talk with other signaling pathways. Structural diversity is achieved by the combination of pore-forming  $\alpha 1$  subunit isoforms with modulatory accessory subunits. To form the  $\text{Ca}_{v1}$  and  $\text{Ca}_{v2}$  VGCC families in neurons, seven  $\alpha 1$  subunit isoforms each combine with one of four different  $\beta$  and one of several  $\alpha_2$ - $\delta$  subunits in hetero-oligomeric complexes (see Catterall et al., 2005 for nomenclature and subunit isoforms). The functional repertoire is further enlarged through alternative splicing (especially of  $\alpha 1$  and  $\beta$  subunits) and the differential susceptibility of channel subtypes to modulation by second messenger pathways (Catterall et al., 2005).

Several mechanisms underlie the modulation of neuronal N-type ( $\text{Ca}_{v2.2}$ ) and P/Q-type ( $\text{Ca}_{v2.1}$ ) VGCCs by neurotransmitters and hormones, and for some of them, the molecular details have been revealed. Activation of G protein-coupled receptors (GPCRs) at the cell surface can affect channel function either through fast, membrane-delimited mechanisms or more slowly via downstream second messengers (Dolphin, 2003; Tedford and Zamponi, 2006; Ikeda and Dunlap, 2007; Lipscombe and Raingo, 2007). Agonist binding to GPCRs can elicit a pertussis toxin-sensitive inhibition of P/Q- and N-type channels that develops and recovers in less than one second, and involves the rapid binding and unbinding of  $\text{G}_{\beta\gamma}$  subunits to well-defined regions of the pore-forming  $\alpha 1$  subunits (Dolphin, 2003; Tedford and Zamponi, 2006). This form of inhibition is voltage dependent and relieved by strong depolarizations (Ikeda and Dunlap, 2007). As a consequence, inhibition is pronounced at slow firing rates but weaker in rapidly firing neurons. Many different GPCRs, such as

muscarinic acetylcholine receptors (mAChRs) and opioid, dopamine, and somatostatin receptors, inhibit calcium currents through this mechanism, which is thought to play a major role in neurotransmitter-mediated control of neurotransmitter release.

Voltage-independent forms of inhibition after GPCR activation also exist. One mechanism involves channel internalization. In dorsal root ganglion (DRG) neurons, internalization of N-type VGCCs is induced either by prolonged activation of ORL1 receptors (Altier et al., 2006; Lipscombe and Raingo, 2006) or after brief activation of  $\text{GABA}_B$  receptors (Tombler et al. 2006). Another form of voltage-independent inhibition of N-type channels by  $\text{G}_i/\text{G}_o$ -coupled GPCRs in DRG neurons requires protein tyrosine kinase phosphorylation in a C-terminal domain (Lipscombe and Raingo, 2007; Raingo et al., 2007). Interestingly, tyrosine phosphorylation occurs within an alternatively spliced region; thus, this type of modulation is only present in exon37a-containing channels that are enriched in nociceptive DRG neurons (Bell et al., 2004). The resulting higher sensitivity of this splice variant to inhibition by neurotransmitters could provide an important mechanism for down-regulation of N-type channel-mediated pain transmission during periods of pain-induced rapid neuronal firing (Raingo et al., 2007).

GPCRs that signal through Gq-like G proteins, such as M1-mAChRs and NK1 receptors, can exploit yet another form of voltage-independent inhibition of N- and L-type current in sympathetic cervical ganglion (SCGs) and cortical neurons. This form of inhibition is sensitive to intracellular calcium buffering, and both onset and recovery are slow (seconds to minutes). It has therefore been referred to as the “slow pathway” (Suh and Hille, 2005; Michailidis et al., 2007). This pathway has attracted special attention because lipids were implicated as effector molecules, thus representing another example of lipid modulation of ion channels and transporters (Wu et al., 2002; Gamper et al., 2004; Suh and Hille, 2005). Although the exact mechanism remains controversial, two distinct hypotheses are the

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Abbreviations used in this paper: ArA, arachidonic acid; DRG, dorsal root ganglion; GPCR, G protein-coupled receptor; mAChR, muscarinic acetylcholine receptor; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; SCG, sympathetic cervical ganglion; VGCC, voltage-gated calcium channel.

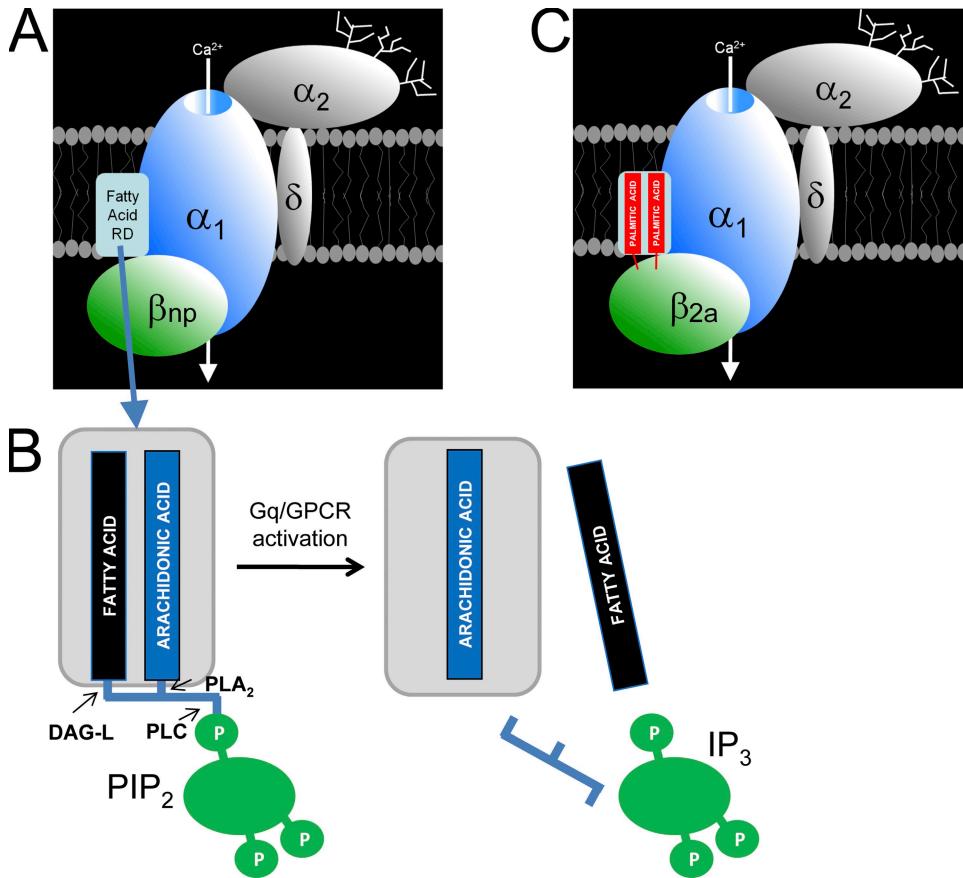
main competitors for resolution: a phosphatidylinositol 4,5-bisphosphate ( $\text{PIP}_2$ ) depletion and an arachidonic acid (ArA) generation hypothesis. The former implies that N-type (and P/Q-type) channel activity is stabilized by  $\text{PIP}_2$ , most likely through binding to a fatty acid/lipid regulatory site near the channel (Fig. 1 A). Inhibition would occur upon PLC-induced breakdown with membrane depletion, and dissociation of  $\text{PIP}_2$  from the channel (Wu et al., 2002; Gamper et al., 2004; Delmas et al., 2005). This is an attractive hypothesis because the type of modulation closely resembles the inhibition of M-current (KCNQ2/3) by M1 mACHR, a major regulator of neuronal excitability (Suh et al. 2006).

A series of reports from the Rittenhouse laboratory provides support for an alternative view, an “ArA generation” hypothesis. This hypothesis predicts that whereas M-current inhibition requires only  $\text{PIP}_2$  breakdown, L- and N-current inhibition in SCGs may depend on additional signaling events downstream of PLC/ $\text{PIP}_2$  (Liu and Rittenhouse, 2003; Liu et al., 2006). They found that the M1 mACHR signaling cascade for N-type (and L-type) current modulation in SCG neurons not only involved activation of  $\text{G}_q$ , but also complete  $\text{PIP}_2$  breakdown by PLC, PLA<sub>2</sub>, and diacylglycerol lipase (Fig. 1 B) (Liu and Rittenhouse, 2003; Liu et al., 2006, 2008). ArA was the most likely downstream effector because different measures reducing ArA availability (PLA<sub>2</sub> gene

knockout, PLA<sub>2</sub> pharmacological inhibition, and ArA scavenging by BSA) attenuated modulation by receptor activation. Moreover, exposure of cells to ArA largely mimicked the typical characteristics of M1 mACHR modulation of native currents in SCG neurons. Although these results favor the “ArA generation” over the “ $\text{PIP}_2$  depletion” hypothesis, these two are not mutually exclusive, as discussed below.

ArA treatment also mimicked another typical feature of M1-mACHR-mediated current modulation in SCG neurons. Depolarization of cells to a wide range of different potentials revealed stimulation of current at more negative (below 0 mV) and inhibition at more positive (above 0 mV) test potentials. It was this dual response that finally provided an important hint to the molecular mechanism of this pathway.

The Rittenhouse laboratory presents two papers (see Heneghan et al. and Mitra-Ganguli et al. in this issue) describing novel findings shedding more light on how N-type channel function is modified by lipids. The key observations were made in a heterologous expression system. When  $\text{Ca}_{v}2.2 \alpha 1$  subunits were expressed with  $\beta 3$  (or  $\beta 1$  or  $\beta 4$ ) subunits, the inhibitory effect at positive potentials of M1-mACHR or NK1 receptor activation or of externally applied ArA was nicely reproduced, but the stimulatory component at negative potentials was absent. Moreover, the currents showed much faster



**Figure 1.** Mechanisms involved in lipid modulation of voltage-gated N- and L-type calcium channels by the slow pathway. The subunit structure of high threshold neuronal calcium channel complexes is illustrated together with a putative fatty acid regulatory domain (RD) that can also bind the palmitic acid side chains present in the N terminus of  $\beta 2a$  subunits. For details see text. PLC, phospholipase C; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; DAG-L, diacylglycerol lipase; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; IP<sub>3</sub>, inositol triphosphate;  $\beta_{np}$ , nonpalmitylated  $\beta$  subunit.

voltage-dependent inactivation during depolarizations than N-type currents in SCGs. This directed attention to  $\beta 2a$  subunits, one of the many  $\beta 2$  subunit splice variants expressed in neurons, well known for its unique capacity to induce slow inactivation of VGCCs. Like all the other  $\beta$  subunits, it binds to a conserved motif (termed AID) within the cytoplasmic loop between the first transmembrane helix of repeat I and the last transmembrane helix of repeat II of the  $\alpha 1$  subunit. The former (IS6) is part of the inner portion of the ion-conducting pathway and participates in channel inactivation. Recent elegant work from the Perez-Reyes (Vitko et al., 2008) and Dan Minor (Findeisen and Minor, 2009) laboratories revealed that the modulatory effects of  $\beta$  subunits are transmitted to the pore by the rigid linker between AID and IS6. Unlike all other known  $\beta$  splice variants,  $\beta 2a$  is palmitoylated at two N-terminal cysteins (Chien et al., 1996). These fatty acids provide a membrane anchor for the N terminus and must restrict conformational flexibility of  $\beta 2a$  (Van Petegem et al., 2004). This restriction may in turn impair the flexibility of IS6 movements and thereby stabilize slow inactivation gating. Surprisingly, when Heneghan et al. (2009) co-expressed  $\beta 2a$  subunits, they not only observed the expected slowing of inactivation of recombinant N-type currents, but also found that receptor activation or ArA treatment caused channel stimulation rather than inhibition, as observed for the other  $\beta$ s. But how could different  $\beta$  subunits stabilize opposite modulatory actions?

From earlier work it was known that current enhancement by ArA occurs from the extracellular side and inhibition from inside (Barrett et al., 2001; Liu et al., 2001). Therefore, Rittenhouse and colleagues reasoned that the differential effects of the cytoplasmic  $\beta$  subunits must result from interaction with the intracellular inhibitory site. The simplest explanation was that  $\beta 2a$  is able to somehow block intracellular inhibition by ArA, thereby unmasking stimulation, whereas  $\beta 1$ ,  $\beta 3$ , and  $\beta 4$  subunits are unable to do so and allow ArA to decrease channel currents. If this explanation is true, the palmitoylation of  $\beta 2a$  was the logical molecular explanation for this difference between  $\beta$  subunits. Accordingly, co-expression of a palmitoylation-deficient  $\beta 2a$  prevented the stimulatory effects of receptor activation and ArA exposure. Therefore, it was reasonable to hypothesize that the anchoring palmitic acid side chains interfere with ArA binding at a channel site located within the reach of the  $\beta 2a$  N terminus (Fig. 1 C). The addition of free palmitic acid also attenuated inhibition. Although supportive, this finding could hardly be taken as strong molecular evidence for the proposed mechanism. However, they correctly interpreted another (at first sight disappointing) experimental observation. When they introduced the palmitoylated N terminus of  $\beta 2a$  into  $\beta 1$ , the resulting chimera more or less reproduced the

$\beta 2a$  behavior on modulation. However, a similar chimeric construct with  $\beta 3$  did not block the inhibitory component effectively. As the docking of all  $\beta$  subunits to  $\alpha 1$  subunits via AID should occur in a similar manner, the most likely explanation was that differences in  $\beta$  subunit size and geometry prevented the precise placement of the palmitic acid side chains in the putative ArA binding pocket by the  $\beta 3$  chimera. To support this hypothesis, they used a more defined approach to disturb the position of the palmitoylated  $\beta 2a$  N terminus. They exploited previously characterized  $\text{Ca}_{v}2.2$   $\alpha 1$  subunit constructs in which the orientation of  $\beta$  subunits relative to  $\alpha 1$  is altered by deleting one or two residues of the putative  $\beta$  sheet in the IS6-AID linker (Vitko et al., 2008). In accordance with their predictions, a minor (only one residue deleted) disturbance of  $\beta$  subunit orientation destabilized the  $\beta 2a$ -mediated block of inhibition, whereas a two-residue deletion completely relieved inhibition and resulted in the expected inhibitory modulation similar to nonpalmitoylated  $\beta$ s.

These findings draw a completely new picture of the role of  $\beta 2a$  subunit palmitoylation. Clearly, it not only serves as a simple membrane anchor like in many other proteins, but it also must be considered as a steric inhibitor of an internal fatty acid regulatory domain for ArA, close to the channel (Fig. 1 C). Overall, this mechanism allows VGCCs to exploit  $\beta$  subunit diversity not only to obtain different gating properties, but also to select between stimulation or inhibition by  $G_q$ -coupled GPCRs. This versatile modulation of VGCCs depends on the relative abundance of  $\beta 2a$  in relation to other nonpalmitoylated  $\beta 2$  splice variants and  $\beta$  subunit isoforms. These ideas and results should prompt further studies to quantify the subcellular distribution of individual  $\beta$  subunits in neurons and to test for dynamic changes of their expression under different physiological and pathophysiological conditions. Because palmitoylation is a reversible process and as such has been reported to modulate chromaffin cell calcium channel function (Hurley et al., 2000), the possibility of neuronal calcium channel fine-tuning by dynamic palmitoylation of  $\beta 2a$  will need further attention.

An important final question remains: How do the present findings affect the controversy between the “PIP<sub>2</sub> breakdown” and “ArA generation” hypotheses? They clearly uphold the latter by providing a plausible molecular mechanism supporting it. However, previous experimental evidence arguing against a role for PLA<sub>2</sub> and ArA (Wu et al., 2002; Gamper et al., 2004) remains valid. Unfortunately, some differences in experimental design between these latter studies (use of the oocyte expression system instead of HEK cells, no use of  $\beta 2a$  in heterologous system, and frog instead of mammalian sympathetic neurons) and those from the Rittenhouse laboratory (use of barium instead of physiological calcium as charge carrier) exist and complicate direct

comparisons. Nevertheless, a unifying hypothesis can be proposed that could serve as a basis for further experiments. Most PIP<sub>2</sub> contains ArA in the sn2 position and another fatty acid (mostly stearic acid) in the sn1 position (Fig. 1 B). Release of ArA is catalyzed by PLA<sub>2</sub>, whereas release in sn1 position is catalyzed by diacylglycerol lipases. The Rittenhouse group has found that both lipases in addition to PLC (which removes the negatively charged IP<sub>3</sub>) are required for M1-mAChR-induced inhibition of N- and L-type channel activity through the slow pathway (Liu et al., 2008). This suggests that PIP<sub>2</sub> stabilizes the channel and does so by occupying the fatty acid regulatory domain with its fatty acid side chains. Tethering to the inositol and glycerol backbones may stabilize a conformation preventing inhibition and stabilizing channel function. However, once all three enzymes have disassembled the PIP<sub>2</sub> molecule (Fig. 1 B), the fatty acid regulatory domain becomes available for inhibition by ArA released from PIP<sub>2</sub> and perhaps other sources. Stable occupation of the site by palmitic acid anchored to  $\beta 2a$  could prevent any inhibitory effects by released ArA. Promoting PIP<sub>2</sub> formation would stabilize channel function by counteracting ArA inhibition, whereas external ArA addition would counteract stabilization by PIP<sub>2</sub>. No information exists regarding a possible interaction between PIP<sub>2</sub> and the palmitic acid side chains of  $\beta 2a$ .

The investigations of Rittenhouse and colleagues further emphasize the role of  $\beta$  subunits as important targets for VGCC modulation. Ras-related RGK family of small GTP-binding proteins can bind to  $\beta$  subunits and thereby inhibit channel activity (Béguin et al., 2001). As another recent example,  $\beta$  subunit interaction with the presynaptic active zone protein RIM induces profound changes of voltage-dependent activation and inactivation gating in Ca<sub>v</sub>2 VGCCs, thus supporting calcium influx for neurotransmitter release (Kiyonaka et al., 2007).

The novel findings described in this issue provide an important first step into the molecular understanding of the modulation of VGCCs by ArA and/or PIP<sub>2</sub>. Hopefully, further work will precisely localize these regulatory domains within one or more of the VGCC subunits. Elegant work on other ion channels, such as the functional interconversion between A-type and delayed rectifier K<sup>+</sup> channels by PIP<sub>2</sub> and ArA (Oliver et al., 2004), provides important motivation for such studies.

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