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REGULATION OF VOLTAGE-GATED Ca^{2+} CHANNELS BY LIPIDS

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Abstract

Great skepticism has surrounded the question of whether modulation of voltage-gated Ca^{2+} channels (VGCCs) by the polyunsaturated free fatty acid arachidonic acid (AA) has any physiological basis. Here we synthesize findings from studies of both native and recombinant channels where micromolar concentrations of AA consistently inhibit both native and recombinant activity by stabilizing VGCCs in one or more closed states. Structural requirements for these inhibitory actions include a chain length of at least 18 carbons and multiple double bonds located near the fatty acid's carboxy terminus. Acting at a second site, AA increases the rate of VGCC activation kinetics, and in $Ca_V2.2$ channels, increases current amplitude. We present evidence that phosphatidylinositol 4,5-bisphosphate (PIP_2), a palmitoylated accessory subunit (β_{2a}) of VGCCs and AA appear to have overlapping sites of action giving rise to complex channel behavior. Their actions converge in a physiologically relevant manner during muscarinic modulation of VGCCs. We speculate that M_1 muscarinic receptors may stimulate multiple lipases to break down the PIP_2 associated with VGCCs and leave PIP_2's freed fatty acid tails bound to the channels to confer modulation. This unexpectedly simple scheme gives rise to unanticipated predictions and redirects thinking about lipid regulation of VGCCs.

Keywords

Ca_V; $cPLA_2$; DAG liapse; G_q; muscarinic receptors; ischemia; fatty acid

1. Introduction

Voltage-gated ion channel activity is exquisitely regulated to maintain accurate flow of information throughout the body allowing us to breathe, run, talk, learn, and remember. There

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is increasing appreciation that lipid molecules regulate and modulate ion channels that interact directly with them in the plasma membrane. Particular interest surrounds lipid regulation of voltage-gated Ca^{2+} channels (VGCCs) since changes in their activity affect not only membrane excitability, but also neurotransmitter release, Ca^{2+}-dependent biochemical events, and activity-dependent gene transcription [1]. Thus, a change in VGCC activity may precipitate acute (msec-sec), short-term (sec-min), and long-term changes (hrs-days). Lipid-induced regulation of VGCC activity is incompletely understood; however there is growing appreciation that phosphatidylinositol 4,5-bis phosphate (PIP_2) and free fatty acids (Fig. 1) play important roles in regulating VGCC activity. For example, PIP_2 increases channel availability to open and promotes slowed gating kinetics, whereas fatty acids exert the opposite actions. In particular, growing evidence indicates that arachidonic acid (AA), a 20 carbon, polyunsaturated fatty acid is liberated from the *sn*-2 position of phospholipids, such as PIP_2 (Fig. 1A), by phospholipases to modulate the activity of pore-forming VGCC subunits of the $Ca_V\alpha_1$ superfamily.

The following review synthesizes current knowledge of the mechanisms used by PIP_2 and AA to regulate VGCC currents. Basic chemistry about PIP_2 and AA in the plasma membrane is presented when deemed helpful. A number of recent, comprehensive reviews thoughtfully address many of the physiological implications of phospholipid [2–4] and fatty acid [5–7] regulation of VGCCs. Where useful, AA's actions will be compared to other fatty acids (Fig. 1B) but not its metabolites. For a thorough account of the effects of fatty acid metabolites on ion channels, see excellent reviews by Spector and Norris and by Meves [6,8,9]. Enzymes bind AA with K_ms in the μM range, indicating that μM concentrations of AA are physiological [10]. At or above 20 μM, AA micelles can form though the exact value varies depending on ionic concentrations [11]. Above the critical micelle concentration, lipids will aggregate into spherical structures in solution where they can exert detergent effects on the lipid bilayer. Therefore we discuss studies where fatty acid concentrations used are less than 20 μM. A number of neurotransmitters that stimulate release of endogenous AA in a variety of cell types also modulate VGCCs. Some controversy exists as to whether released AA participates in transmitter modulation of VGCCs or whether simple dissociation of PIP_2 is sufficient for decreased channel activity. Therefore we discuss the controversy surrounding the putative roles that PIP_2 and AA may play in mediating VGCC modulation by G_q-coupled receptors (G_qPCRs). A unifying model for VGCC modulation is presented that takes into account data from PIP_2 and AA studies. Lastly we discuss the molecular significance of lipids in regulating VGCC activity.

2. Exogenously applied AA inhibits VGCC currents by stabilizing closed or inactivated conformations

The pore-forming $Ca_V\alpha_1$ subunits arise from a 4-fold repeat of 6 transmembrane segments with a pore-loop inserted between S5 and S6 (Fig 2A). Cytoplasmic loops link the four domains into a single polypeptide. Recent crystal structures of voltage-gated K_v channels serve as a rough template for interpreting very basic structure-function studies of VGCCs. As with K_v channels, S1-S4 form a voltage sensor unit with S4 containing multiple positively charged residues that move in response to changes in membrane potential. S5 and S6 form the pore of the channel with S6 serving as its inner lining. S6 contains amino acid residues responsible for channel opening, closing and certain forms of inactivation [12]. However, members within the VGCC family exhibit diverse gating behavior making it difficult to generalize about how their different structures affect activity.

Nevertheless, from mutagenesis and kinetic studies there is general agreement that by mechanisms not yet well understood, movements of the voltage sensor somehow cause each S6 to flex away from one another, to open VGCCs. Closed times, measured from single channel

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patch-clamp recordings, distribute around several time points indicating that VGCCs have multiple closed states. Some combination of each voltage sensor moving through the membrane and S6 flexing may account for the multiple closed states [13]. Moreover when cells are held very negative, the four voltage sensors reside in their most internal position within the membrane. Upon depolarization, channels take a measurable amount of time to transition through these multiple closed conformations to open; this time is referred to as the first latency. An altered first latency indicates changes in stability of these closed conformations. As VGCCs transition through the partially activated closed states during membrane depolarization, they will undergo slow forms of voltage-dependent inactivation that develop over 100's to thousands of msec. These slow forms of inactivation, often referred to as C-inactivation for K^+ channels, are thought to occur following reorientation of residues within or near the outer pore region. VGCCs also undergo faster forms of inactivation, developing over tens to several hundreds of msec, where the mechanism underlying inactivation appears to vary depending on the type of VGCC. For some VGCCS, cytoplasmic loops appear to interact with the inner pore to block the current, giving rise to fast inactivation. Additionally fast forms of inactivation for some VGCCs can exhibit characteristics of C-type inactivation, underscoring the complexity of VGCCs' biophysical properties. For more in depth discussion of VGCC gating see reviews by [12–16].

Exogenously applied AA inhibits currents of both native and recombinant VGCCs from all three families of α_1 pore-forming subunits Ca_V1-Ca_V3. Where examined, all VGCCs exhibit sensitivity to AA with an IC_{50} within 1–10 μM [17–25]. Ca_V1 (L-channel) and Ca_V2 (N-, P/Q-and R-channels) pore-forming subunits exist in macromolecular complexes with accessory subunits $\alpha_2\delta$ and β, which help increase functional expression and tune the channels' biophysical properties [26]. The β-subunit also aids in trafficking these channels to the cell membrane [27]. Despite the fact that multiple subunits form these VGCCs, they are referred to by the name of their $Ca_V\alpha_1$ pore-forming subunit. In contrast, Ca_V3 (T-channel) subunits give rise to recombinant current without a requirement for accessory subunits [28]. This latter finding suggests that whether direct or indirect, AA acts on Ca_V pore-forming subunits. Moreover, AA appears to alter the gating of VGCCs but not channel permeation. Details of AA's actions on each VGCC family are presented below.

2.1 T-type Ca^{2+} Channels

AA inhibits T-current in adrenal zona fasciculate cells [29], rat osteoblast cells [30], NG108-15 cells [31] and currents from all three recombinant T-channels ($Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$) [18,20,21] though $Ca_V3.2$ exhibits a greater magnitude of inhibition than $Ca_V3.1$ or $Ca_V3.3$ [18]. AA itself appears to modulate Ca_V3 channels since blocking AA metabolism has no effect on the magnitude of current inhibition. Additionally, AA can robustly inhibit T-currents from inside-out, ripped-off patches consistent with a direct effect [18,21]. No change in unitary conductance was found, but rather, an increase in sweeps with no activity occurred following AA [21]. Consistent with this finding, at the whole-cell level AA could inhibit channels in the closed state. A Hill coefficient of 1.6 best describes inhibition of Cav3.1 by AA suggesting that at least two AA molecules cooperatively interact with one channel [18,20,21]. A detailed study by Chemin et al. demonstrated that other unsaturated free fatty acids can suppress Ca_V3 currents while saturated free fatty acids have no effect [18]. Fatty acid inhibition of T-current has a structural requirement of at least 18 carbons in chain length. Potency of inhibition increases with increasing numbers of *cis* double bonds and with placement of the double bonds near the carboxyl group [18].

Further kinetic studies examining the mechanism of inhibition indicate AA also increases the voltage-sensitivity of slow forms of inactivation of all three T-channels [18,20,21]. Talavera et al. (2004) found that the onset of current inhibition and changes in slow inactivation were

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different indicating AA has two different actions on $Ca_V3.1$. Changes in fast forms of inactivation do not appear to underlie inhibition. Talavera et al. came to this conclusion through studies using mutant $Ca_V3.1$ channels with decreased fast inactivation. Most notably, a point mutation in IIIS6 of $Ca_V3.1$ (M1510I) leads to currents that inactivate with a time constant 4-fold greater than for wt channels [32]. Despite the slower inactivation kinetics, AA inhibits M1510I currents more rapidly and with an approximately 10-fold lower IC_{50} (~0.3 μM) than wt currents (Fig. 2A) [21]. AA has little effect on the voltage-dependence of activation [18, 20,21]; however, a small, but significant negative shift in the foot of the activation curve in $Ca_V3.1$ currents and a small change in the slope of the activation curve for $Ca_V3.2$ occurs following AA that can be accounted for by changes in the rate constants between closed states [20,21]. Thus, AA appears to decrease channel availability by stabilizing closed conformations and also shifts the steady-state inactivation curve more negative.

2.2 L-type Ca^{2+} Channels

AA inhibits L-current in skeletal ($Ca_V1.1$) [22,36], cardiac ($Ca_V1.2$) [17,25,37] and smooth muscle myocytes ($Ca_V1.2$) [38], SCG neurons (primarily $Ca_V1.3$) [23,39–41], recombinant $Ca_V1.3$ current expressed in HEK 293 cells [42]; and in photoreceptors (primarily $Ca_V1.4$) [19]. In hippocampal neurons, which express both $Ca_V1.2$ and $Ca_V1.3$ channels, AA exerts similar inhibitory actions on the whole-cell L-current [43]. At the single channel level, AA inhibits unitary L-channel activity in SCG neurons by increasing mean first latency and mean closed time [41]. Additionally increased numbers of sweeps with no openings are observed, raising the possibility that AA also stabilizes inactivated states. Conductance and mean open time are unaffected by AA [41].

At the whole-cell level, AA increases the amount of $Ca_V1.3b$ current inactivation at positive test potentials, but does not alter the voltage profile of inactivation [35]. This form of $Ca_V1.3b$ has a truncated C-terminus ruling out AA binding to G-protein and calmodulin binding sites. Findings from three additional experiments, designed to minimize L-current inactivation, indicate AA decreases current independently of $Ca_V1.3b$ inactivation [35]. 1) When $Ca_V1.3b$ is held at −90 mV, few channels inactivate; most reside in the closed state whereas at −60 mV channels reside in a partially activated closed state become susceptible to slow forms of inactivation. Holding the membrane potential more negative (−90 mV versus −60 mV) approximately doubles $Ca_V1.3b$ current inhibition by AA when cells were stepped to −10 mV every four seconds, the potential where maximal inward current normally occurs [35]. 2) In a similar experiment, current inhibition still occurs when L-channels are held closed during application of AA [19,35,38,42], consistent with the hypothesis that AA stabilizes closed states. 3) Shortening the test potential duration from 40 ms to 10 ms decreases the number of channels that will inactivate. Under these conditions the magnitude of inhibition increased. In each of these three experiments the voltage protocol was manipulated to increase the time the channel spent in a closed state while minimizing the probability that channels would transition to inactivated conformations. In each case, inhibition remained the same or increased in magnitude.

These data from recombinant channels best fit a model where AA stabilizes channels in a deep closed state, rather than an inactivated state, and match whole-cell and single channel findings of native L-currents in SCG neurons [23,41]. However, in other cell types, AA shifts the voltage-sensitivity of inactivation for L-current more negative [25,37,38,44] similar to the schematic shown in Figure 2C. Amino acid residues found in transmembrane S5 and S6 segments regulate opening, closing and slow forms of inactivation [14,32,45–48]. Sequence variation in these regions among L-channels may explain these differences in response to AA.

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2.3 N-type Ca^{2+} Channels

AA inhibits native N-current in SCG neurons [23,39,41,49] and recombinant N-current ($Ca_V2.2$) expressed in HEK 293 cells [39,50]. Other members of the Ca_V2 family [$Ca_V2.1$ (P/Q) and $Ca_V2.3$ (R)] have not been tested for sensitivity to AA. Native N-channels display distinct patterns of endogenous, heterogeneous activity called modes [51,52]. AA alters the frequency of occurrence of particular modes to decrease unitary N-channel activity in cell-attached patches that contain one channel (Fig. 3A). AA decreases open probability (P_o) by increasing the percentage of sweeps with no openings (nulls) and the number of null sweeps that cluster together [41]. AA stabilizes a null mode but does not alter modes with amplitude or open time changes. However, truncated closings (the last closing during the test pulse) increase 2–3-fold in duration, indicating channels enter a non-conducting state (closed or inactivated). Increases in first latency usually occur when channels transition through closed conformations to the open state more slowly than normal. First latency increases approximately 3-fold, indicating that AA stabilizes one or more intermediate closed states rather than an inactivated state.

N-current comprises 80–90% of the whole-cell current in rat SCG neurons [53]. Kinetic analyses of these whole-cell currents revealed AA selectively decreases the frequency of non-inactivating gating with little change in fast inactivation (Figs. 3C–D). These findings are consistent with the single channel results where AA stabilizes one pattern of activity, the null mode, while specifically decreasing another, the noninactivating mode (Figs. 3A–B). Additionally whole-cell studies showed that AA increased the amount of holding potential-dependent inactivation at positive voltages [23], similar to that observed with recombinant $Ca_V1.3b$ currents (Fig. 2B). This increase in inactivation may also contribute to the observed increased incidence of the null mode at the single channel level. However, no shift in voltage-sensitivity of inactivation was observed [23]. This lack of voltage dependency may be due to AA stabilizing a closed state that is susceptible to undergoing transitions to inactivated conformations [54]. Taken together, these findings support a model where AA may stabilize a closed conformation of the N-channel that normally promotes inactivation. Alternatively, if the rate of recovery from inactivation is also slowed, channels may accumulate in an inactivated conformation. Future biophysical studies combined with mutagenesis should allow more precise characterization of the mechanism of N-channel inhibition by AA.

In summary, the similar actions of AA across VGCC families suggest a common mechanism for inhibition. N-channels, as with T- and L-channels, exhibit changes in closings following exposure to AA. Additionally, AA appears to have a second though more varied effect on all three channels where kinetic changes in inactivation occur. Consistent with the idea of common mechanism, fatty acid inhibition of VGCC activity has similar structural requirements: 1) a minimum carbon chain length of 18 carbons; 2) the magnitude of inhibition increases with number of carbons 3) polyunsaturated bonds; 4) and double bond locations close to the carboxy terminus [18,19,23,33–35,39].

3. AA exerts biphasic actions on VGGCs

Despite the attention given to inhibition, reports of biphasic effects suggest AA modulates VGCCs at more than one site. In addition to inhibiting VGCC currents (Fig. 3C), AA acts at a second, distinct site to enhance N-current at negative test potentials [23,39,49]. Enhanced N-current exhibits an increased rate of activation that correlates in time with a leftward shift in voltage-sensitivity of activation [39]. N-current enhancement by AA is manifest in the current-voltage relationship at negative test potentials (such as depolarizations to −10 mV), whereas N-current inhibition dominates the modulation observed at positive test potentials (such as depolarizations to +10 mV). ETYA, myristic acid (Fig. 1B), and palmitic acid (5 μM) enhance, but do not inhibit N-current, demonstrating that the two effects have different fatty acid

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specificities [23,39]. When alternating test pulses to −10 mV to monitor enhancement and to +10 mV to monitor inhibition, the opposing effects can be observed in the same time courses. The onset of enhancement develops significantly faster than inhibition, suggesting that the opposing effects of AA occur by separate mechanisms [23,39].

Consistent with this possibility, the two actions separate with pharmacological manipulation [39]; inhibition appears to occur intracellularly or in the inner membrane leaflet while enhancement may occur extracellularly or in the outer membrane leaflet. When included in the pipette solution, bovine serum albumin (BSA) dialyzes into cells and serves as an AA scavenger since it binds AA quite effectively [55]. Dialyzing SCG neurons with BSA has no effect of its own on control currents nor does it alter enhancement following exposure to AA. However its presence minimizes N-current inhibition by AA, suggesting that the opposing effects have different sites of action. This hypothesis was further tested with bath application of AA covalently bound to coenzyme A. Very little of AA-coenzyme A (AA-CoA) crosses cell membranes because of its hydrophilic nature. When introduced into the bath, AA-CoA enhanced N-current at negative potentials but had no inhibitory effects at positive test potentials. Thus, intracellular regions of N-channels appear to confer inhibition, whereas enhancement by AA may be mediated extracellularly [23,39]. Alternatively, enhancement may occur intracellularly but exhibit a higher affinity for free AA than the site of inhibition [23].

Two sites of action may exist for other types of VGCCs. In cardiac myocytes, AA produces both a hyperpolarizing shift in L-current activation and inactivation as well as a reduction in the maximum slope conductance [25]. In smooth muscle, myristic acid (Fig. 1B) enhances L-current at negative potentials [38], with a similar current-voltage profile as N-current modulation by myristic acid [39]. Other fatty acids such as palmitic acid stimulate L-current in pancreatic α-cells [56] while palmitic and oleic acids stimulates L-current in pancreatic β-cells [57,58]. In contrast, only inhibition by AA has been observed for L-current in skeletal muscle myocytes ($Ca_V1.1$), smooth muscle myocytes (primarily $Ca_V1.2$) and SCG neurons ($Ca_V1.3$) [23,38,49]. Similarly, an increased rate of activation occurs in T-currents following AA with no increase in current amplitude [21,42]. Thus, inhibition by AA may dominate VGCC activity, obscuring any appearance of the ongoing enhancing effects of AA [39]. When matched with conditions used in earlier studies, these results taken together provide an explanation for AA's seemingly paradoxical actions on VGCC currents [38,44,59–62]. Future mutagenesis studies should resolve whether homologous, distinct sites exist across the three VGCC families that mediate enhancement and inhibition by AA.

4. Evidence for direct AA binding to channels

Once liberated, AA binds to a number of cytosolic proteins indicating that it can interact directly with specific proteins in either a lipid environment or in the more hydrophilic cytosolic environment [11,44,63–65]. Specific binding of AA to VGCCs remains difficult to demonstrate because of the overall size of channel proteins and the large number of hydrophobic residues that comprise the transmembrane domains that normally contact the lipid bilayer. Examples of proteins that bind AA include the cystic fibrosis antigen complex S100A8/S100A9 [66], certain forms of protein kinase C (PKC) [67], the catalytic subunit of protein phosphatase 2B (PP2B or calcineurin) [68], PP5 [69], fatty acid binding protein [70], and BSA [71]. In the C-terminal tail of S100A9, three consecutive histidine residues were identified for AA binding [66]. AA binds to BSA at multiple sites with high affinity (K_d, 62 nM) [71]. Surprisingly no sequence homology or obvious three dimensional structure is shared among the sites [72–74]. AA binding sites of other voltage-gated ion channels also remain mostly undefined; however, mutational studies have identified key residues in the pore region of the Na^+ channel $Na_V1.5$. A single point mutation (N406K) in IS6 (Fig. 2A) minimizes fatty acid inhibition of channel activity [75]. In contrast, AA inhibits a double mutant (L409C/A410W

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in the S6 of domain I), suggesting that multiple amino acids contribute to channel sensitivity to fatty acids. An asparagine is found in a homologous site in S6 of domains I–IV in all Ca_V channels, suggesting a possible common site of interaction with AA. In contrast to the possible common site of interaction for VGCCs and $Na_V1.5$, three different potential sites of interaction with AA have been identified for K+ channels. Hamilton et al. (2003) identified two amino acids (Thr250 and Val275) in the pore-lining region of the human intermediate conductance, Ca^{2+}-activated K^+ channel (hIK1) as key residues for current inhibition by AA [76] while the amino terminus of ROMK1 and the carboxy terminus of Kir3 confer inhibition [77,78]. In contrast, the carboxy terminus of TREK-2 confers enhancement by AA [79].

Thus, a common consensus sequence for AA binding may not exist across different protein families. Nevertheless, since AA elicits similar changes in L-, N- and T-channel activity, AA may act at one or more shared homologous sites. AA inhibits T-current recorded in ripped off, inside-out patches [18] where most second messenger systems are absent, ruling out a number of signaling molecules and suggesting direct binding. Moreover, T-channels require no accessory subunits for current expression, yet AA inhibits T-current similarly to native and recombinant Land N-currents [23,35,39], consistent with AA binding to homologous sites on pore-forming subunits. Future mutagenesis studies should reveal whether VGCCs have a common consensus site for AA binding and/or conferring modulation.

In the meantime, biochemical studies provide tantalizing clues as to regions of Ca_V1 that may interact with AA. Three amino acids in the pore-region of L-channels have been identified by extensive mutagenesis and chimera studies as critical for binding of dihydropyridines (DHPs) [80,81], a class of L-channel agonists and antagonists (Fig. 2A). Docosahexaenoic acid (C22:6), but not AA, inhibits the binding of the DHP antagonist nitrendipine in cardiac myocytes [82,83]. AA displaces the specific binding of the radiolabelled DHP antagonist PN200-110 with an IC_{50} of approximately 6 μM in rabbit skeletal muscle membrane assays [84] and decreases PN200-110 binding and $^{45}Ca^{2+}$ flux by 50% in skeletal muscle T-tubules [22]. Blocking lipoxygenase activity has no significant effect on displacement of PN200-110, consistent with AA, rather than a metabolite, competing with DHP antagonist for binding to $Ca_V1.1$. Additionally, DHPs augment the binding of other Ca_V1 channel antagonists through allosteric interactions [81], a profile similar to that observed with AA where AA augments the binding of radiolabelled diltiazem and D888, L-channel antagonists with binding sites distinct from DHPs [84]. These findings identify the DHP binding site as one potential site for AA interaction with Ca_V1 channels. Whether AA interacts with analogous sites in the pore region of Ca_V2 and Ca_V3 channels awaits testing; however it is an attractive idea since high concentrations of DHPs will inhibit native N-current [85].

The canabinoids anandamide and 2-AG share structural similarities to AA and their binding to cannabinoid GPCRs results in modulation of VGCC activity [86]. Additionally, anandamide inhibits P-type Ca^{2+} currents in the cerebellum [87], L-current in T-tubule membranes [36], N-current in SCG neurons [88] and recombinant T-current [18,89] independently of receptor activation. Anandamide produces a greater inhibition of all three ($Ca_V3.1$, $Ca_V3.2$, $Ca_V3.3$) T-currents than AA [18]. As reported for AA, anandamide competes with DHP agonist/antagonist binding to L-channels [82,83], possibly by direct competition [84]. Together these findings identify the pore-region as a potential binding site for AA or alternatively as important for conferring Ca^{2+} channel modulation by AA [34].

5. Palmitoylation of the β2a subunit alters VGCC gating and modulation by AA

Studies using recombinant L- or N-channels revealed that the magnitude of current inhibition by AA varied with different β-subunits [50,90]. Whole-cell currents from $Ca_V1.3b$ coexpressed

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with β_{2a} showed approximately half the inhibition by AA than when coexpressed with either β_1, β_3 or β_4 [90]. Moreover, following AA the time to peak current decreased in channels coexpressed with β_1, β_3 or β_4 whereas no change in activation kinetics was observed with β_{2a}. Heterogeneous expression of β-subunits may explain the varied responses to AA by native $Ca_V1.2$ channels. Interestingly, AA inhibited currents from $Ca_V2.2$, coexpressed with β_1, β_3 or β_4, whereas coexpression with β_{2a} resulted in sustained *enhancement* of approximately 2-fold at negative test potentials [50]. Heterogeneous association of β-subunits with $Ca_V2.2$ may explain the biphasic action of AA in SCG neurons of enhancement of N-current at negative test potentials and inhibition at positive test potentials [23,39]. Thus, depending on the β-subunit expressed with either L- or N-channels in a cell type, the profile of AA modulation may differ.

Notably β_{2a} differs from β_1, β_3 and β_4 in that it is uniquely palmitoylated at cysteine 3 and 4 of its N-terminus [91]. Channels coexpressed with palmitoylated β_{2a} exhibit noninactivating currents and a rightward shift in the voltage sensitivity of slow forms of inactivation [35,92–100]. By tethering β_{2a} to the membrane, its movements as well as those of the intracellular loops that interact with β_{2a} are impeded. Restricting β_{2a}'s movements results in noninactivating Ca_V1 and Ca_V2 currents [94,101]. Coexpression of a mutant depalmitoyated β_a, which has had cysteine 3 and 4 substituted with serine (β_aC3,4S), results in N-current inhibition rather than enhancement by AA [50]. This finding raises the possibility that the fatty acid palmitoyl groups of β_a disrupt N-current inhibition by exogenously applied AA. When palmitoylation was transferred to β_b by substituting the 16 amino terminus of β_a for β_b's amino terminus while the rest of β_1 remained unchanged, AA no longer inhibits currents of $Ca_V2.2$ coexpressed with the palmitoylated β_a β_1 chimera. Instead, these currents exhibit enhancement at negative test potentials similar to wt β_a. This result suggests that the palmitoyl groups confer the block of inhibition.

One consequence of tethering β_{2a} is that the currents from $Ca_V2.2$ coexpressed with β_{2a} exhibit little inactivation compared to currents from $Ca_V2.2$ coexpressed with β_1, β_3 or β_4. Since both inactivation and modulation are different in β2a-containing channels, the question arises of whether the dramatic slowing of channel inactivation rather than from palmitoylation underlies β2a's effects. Indeed, depalmitoylated β_{2a}C3,4S containing L- and N-channels exhibit increased inactivation as well as inhibition by AA [35,102]. In other words, the palmitoyl groups may block inhibition – not by competing for the same putative binding site as AA – but rather by keeping channels from inactivating. Though AA's primary action appears to stabilize channels in a closed conformation, AA also promotes channel inactivation. Since the palmitoyl groups restrain channels from inactivating, perhaps no inhibition can occur. However, several pieces of data indicate that differences in channel inactivation cannot account for the ability of palmitoylated β2a to block inhibition [35]. First, when cells expressing $Ca_V1.3b$ and depalmitoylated β_{2a}C3,4S are preincubated with free palmitic acid, inhibition by AA is lost while fast inactivation remains. Second, attaching the transmembrane protein segment CD8 to β_{2a}C3,4S rescues noninactivating kinetics [97,98]. Despite the rescued non-inactivating kinetics, coexpression of CD8-β_{2a}C3,4S with $Ca_V1.3b$ exhibits robust inhibition by AA. Third, the β2e splice variant contains a unique hydrophobic N-terminus tail that may insert into the membrane. When coexpressed with either $Ca_V1.2$ or $Ca_V1.3b$, the L-current exhibits little inactivation [35,103], yet is robustly inhibited by AA similar to CD8-β_{2a}C3,4S. These studies indicate that free palmitic acid or the palmitoyl groups of β_{2a} may competitively block inhibition of $Ca_V1.3$ by AA independently of a channel's ability to undergo fast inactivation. Taken together, these L- and N-channel studies with β-subunits reveal a potentially novel role for a palmitoylated protein. The palmitoyl groups ofβ_{2a} don't simply tether the subunit to the membrane but rather may interact with VGCCs to block or interfere with AA's binding site that confers current inhibition (Fig. 5A). Whether other proteins such palmitoylated syntaxins, G-proteins, or scaffolding proteins might also interact with VGCCs

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to alter their gating remains to be tested. Critical to developing this model will be further experiments that test whether certain channel residues change the response to AA and to palmitoylation.

6. PIP_2 exerts actions that oppose AA on VGCC Currents

Currents from Ca_V1 and Ca_V2 channels decrease or "run down" irreversibly over time from ripped off patches of membrane. This observation has led to the hypothesis that critical intracellular components facilitate channel gating, but are lost upon ripping off the patch. What that critical component might be remained elusive until a study by Wu et al., [104] found that application of exogenous PIP_2 slowed rundown of currents from giant, inside-out patches of *Xenopus* oocyte membrane expressing $Ca_V2.1$ (P/Q), $\alpha_2\delta$, and β_4 subunits. In contrast antibodies against PIP_2 accelerated rundown. Similar decreases in rundown were obtained with $Ca_V1.2$ and $Ca_V2.2$ channels, expressed in inside-out oocyte membrane patches following PIP_2 application [105,106] or with whole-cell N-current when dialyzing the PIP_2 analog diC8-PIP_2 into SCG neurons [106]. When conditions were manipulated to increase endogenous PIP_2 levels, currents were maintained or enhanced. Similarly in portal vein myocytes whole-cell L-current increased when dialyzed with 1 μM PIP_3 or $PI(3,4)P_2$ but not $PI(4,5)P_2$ suggesting more specific structural requirements for enhancement [107]. Additional testing may provide a more complete profile of specificity for each VGCC by different phosphatidylinositols.

In contrast, conditions that decreased endogenous PIP_2 levels caused currents to run down more rapidly than observed under control conditions [104,108]. Interestingly, the presence of active catalytic subunit of protein kinase A (PKA) also decreased rundown of $Ca_V2.1$ currents. PKA's effects were not additive with exogenously applied PIP_2 [104]. These findings suggested that PIP_2 may directly associate with recombinant channel protein preventing rundown by maintaining channel availability. Wu et al [104] further found that PIP_2 has a second action where it *decreases* $Ca_V2.1$ currents by promoting reluctant gating. Positive test pulses minimize this effect of PIP_2. Slowed activation kinetics accompanies the voltage-dependence of PIP_2's actions similar to the modulation profile of $Ca_V2.1$ currents by $G\beta\gamma$ of pertussis-toxin sensitive G-proteins [109]. Wu et al. [104] hypothesized that one binding site stabilized $Ca_V2.1$ channels, increasing their availability to open and thus called it the "S" site, whereas they named the second site that confers reluctant gating the "R" site. The actions of PIP_2 appear the opposite of AA: AA decreases availability of channels to open and also acts at a second site to increase activation kinetics promoting willing gating. This mirror image of action though antagonistic suggests that PIP_2 and AA may act at similar sites.

The Yang lab has performed over 85 point mutations in the four S6 segments of $Ca_V2.1$ and characterized current amplitude and kinetic activity of these mutants [46,105]. Mutating one particular residue, an isoleucine located at the intracellular end of IIIS6 to histidine (I1520H) or aspartate (I1502D), significantly decreased rundown whereas point mutations in homologous positions in the other three domains had so significant effect [105]. Similarly homologous point mutations in domain III of L- and N-channels to histidine or aspartate also slowed rundown (Fig. 2A). Exposing mutant channels to PIP_2 had no further effect; however the mutant channels still required PIP_2 since their currents rapidly ran down when endogenous PIP_2 was sequestered by exposing the membrane patches to a positively charged peptide sequence from myristolated alanine-rich protein kinase C substrate (MARCKS). MARCKS sequesters PIP_2 through nonspecific electrostatic interactions between the negative charges of the phosphorylated inositol headgroup [110]. These findings raise the possibility that PIP_2 may normally interact with positively charged amino acids that are spatially near IIIS6. The C-terminus of $Ca_V2.1$ interacts with PIP_2 as well as other phospholipids such as phosphatidic acid in lipid-protein overlay assays consistent with direct interaction between pore-forming

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subunits and phospholipids [111]. Determining the precise locations of PIP_2 interaction sites remains an open and challenging question since complex interactions among the intracellular loops appears to occur [112–114]. Potential PIP_2 interactions with Ca^{2+} channel auxillary subunits have not been tested.

7. Physiological implications of VGCC modulation by lipids

A number of neurotransmitters including acetylcholine, dopamine, and serotonin, hormones and growth factors bind to GPCRs that stimulate phospholipases to liberate fatty acids, such as AA, from autonomic end organs and from nerve cell membranes [115–120]. AA release following muscarinic stimulation has been examined most extensively where AA is primarily liberated from inositol phospholipids. This relationship raises the question of whether neurotransmitters regulate PIP_2 and AA levels to modulate VGCC activity. Muscarinic modulation of L- and N-current by a slow signaling pathway [121–124], first described in SCG neurons (see Hille 1994[125] and Suh & Hille 2005 [126] for review), mimics current enhancement and inhibition by exogenously applied AA [23,39,49,127,128]. However, whether endogenously released AA plays a role in modulating VGCC currents following muscarinic receptor activation is controversial. Here, we discuss the evidence for and against endogenously liberated AA downstream of G_qPCRs mediating VGCC current modulation.

7.1 AA participation in Ca^{2+} current modulation by G_qPCRs is controversial

The muscarinic agonist oxotremorine-M (Oxo-M) inhibits L- and N-current in SCG neurons by the slow pathway via M_1 muscarinic receptors (M_1Rs), G_q and metabolism of PIP_2 by phospholipase C (PLC) [49,104,106,129–131]. Additional phospholipid breakdown appears to participate in current modulation since decreasing AA release by pharmacologically antagonizing PLA_2 activity with oleyloxyethyl phosphorylcholine (OPC) or 7,7-dimethyl-5,8-eicosadienoic acid (DEDA), minimized VGCC current inhibition in SCG and cortical (Fig. 5C) neurons [49,127,128,132]. This slow pathway contrasts the fast, voltage-dependent N-current inhibition by direct G-protein binding to the pore-forming subunit [113]. OPC and DEDA have no inhibitory effects on this fast pathway [133,134].

Little free AA is found in cell membranes. Instead AA is situated in phospholipids of the inner leaflet of the cell membrane. Acyl transferases covalently link AA to the C2 (also referred to as the *sn*-2) carbon of the glycerol backbone of phospholipids, whereas a saturated or mono-saturated fatty acid normally occupies the *sn*-1 position [135]. 80% of PIP_2 has AA in the *sn-2* position [136] (Fig. 1A). Phospholipase A_2 (PLA_2) cleaves fatty acids from the *sn*-2 position of phospholipids, with lysophospholipid arising as the other by-product. Group IVa PLA_2, a cytoplasmic, Ca^{2+}-sensitive PLA_2, also called $cPLA_2$, exhibits selectivity specifically for AA in the *sn*-2 position of phospholipids [137]. $cPLA_2$ associates with membranes by binding to PIP_2 via a C2 domain with an effective increase in enzyme activity [137]. Phosphorylation by ERK1/2 acutely activates $cPLA_2$ following G_qPCR stimulation. These same receptors are associated with phosphatidylinositol metabolism, suggesting that $cPLA_2$ *in vivo* may exhibit some preference for cleaving AA from PIP_2. However careful studies with purified or recombinant $cPLA_2$ have not been performed to fully understand its substrate specificity. PLA_2 will also cleave AA from phosphatidic acid. When antibodies were dialyzed into SCG neurons as functional antagonists of slow pathway modulation, $cPLA_2$ antibodies but not non-immunized IgG or $iPLA_2$ or $sPLA_2$ antibodies minimized inhibition by Oxo-M raising the intriguing possibility that in neurons, $cPLA_2$ may act to liberate AA from PIP_2 to inhibit VGCC activity.

The finding that $cPLA_2$ may participate in M_1R inhibition of L- and N-current challenged a model, illustrated in Figure 4, where dissociation of PIP_2 from VGCCs and subsequent breakdown by PLC is not only necessary but also sufficient for observing inhibition [2,104,

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106]. The Hille lab originally proposed this "PIP_2" model for M-current modulation by M_1Rs. In this model, PIP_2 molecules cycle between binding and dissociating from the KCNQ proteins that give rise to M-current. PIP_2 association increases channel availability to open. Following stimulation of M_1Rs, activated PLC cleaves the inositol head group from unbound PIP_2 [138–141]. Consequently less PIP_2 is present to rebind to channels resulting in decreased M-current. Whether PLA_2 participates in M-current modulation was examined by pharmacologically antagonizing PLA_2 but no adverse effect was found [127,142,143]. Additionally small molecules and various kinases were tested but were also found to play no role in current inhibition [125,126] supporting the idea that PIP_2 breakdown by PLC is sufficient for its modulation by M_1Rs. Because L-, M- and N-current inhibition by the slow pathway in both primary neurons and recombinant systems requires M_1Rs, G_q and PLC [49, 104,106,126,[127],138,140–142,144], the Hille lab and others proposed that the same signal transduction pathway similarly modulates all three currents.

Consistent with the PIP_2 model, Gamper et al. [106] reported no effect of OPC following a 2 min incubation period on N-current inhibition by Oxo-M, when recording from perforated patches of SCG neurons. These findings contrasted whole-cell studies where a 2 minute preincubation with the same OPC concentration resulted in a loss of inhibition by Oxo-M [49]. Gamper et al. [106] suggested that the differences observed with OPC, most likely reflect variations in experimental design rather than in the biology of the system. Similarly, Bannister et al. [145] found that the PLA_2 antagonist quinacrine had no effect on slow pathway inhibition of recombinant L-current ($Ca_V1.2$); however this compound poorly antagonizes $cPLA_2$ [146]. Lastly, Lechner et al. [147] found that inhibition of presynaptic currents by bradykinin was blocked when PIP_2 breakdown was antagonized, yet remained normal when PLA_2 was antagonized with DEDA. In each study, no control experiments were presented that documented selective $cPLA_2$ antagonism, nevertheless these studies raised questions surrounding $cPLA_2$'s role in the slow pathway of VGCC currents.

In a fourth study the PIP_2 analog, diC8-PIP_2 was dialyzed into SCG neurons to effectively expose the VGCCS to unlimited amounts of PIP_2 agonist. Under these conditions, minimal Ca^{2+} current inhibition by Oxo-M occurred, suggesting that if enough endogenous PIP_2 or exogenously applied diC8-PIP_2 remains available to bind to VGCCs, no inhibition will occur [106]. DiC8-PIP_2 lacks the normal fatty acid chains associated with PIP_2, e.g., AA and stearic acid in the *sn*-1 and *sn*-2 positions respectively. Thus diC8-PIP_2 might also act as a substrate competitor of $cPLA_2$, antagonizing AA release from PIP_2 and as a consequence, minimal current inhibition would occur. While this experiment highlighted the importance of PIP_2 in VGCC gating, it did not rule out a requirement for PLA_2 during slow pathway stimulation, leaving $cPLA_2$'s role uncertain.

Subsequent biochemical, imaging, and genetic approaches pointed to a prominent role for $cPLA_2$ activity during L- and N-current inhibition by the slow pathway. First, $cPLA_2$ protein was acutely phosphorylated in SCG neurons following exposure to muscarinic agonist, but blocked in the presence of the MT-7 toxin, a M_1R antagonist, demonstrating its acute activation by M_1Rs [127]. Second, including BSA either in the pipette or bath solution to limit AA levels, antagonized L- and N-current inhibition [49,127]. Moreover, when the BSA-containing bath solution was analyzed for fatty acid content by GC-MS, free AA levels were found to increase approximately 2-fold following ganglion exposure to Oxo-M documenting muscarinic stimulation of AA release from SCG. Most notably, L- and N-current inhibition by Oxo-M, was lost in $cPLA_2^{-/-}$ SCG neurons [127,128]. No significant difference in control current amplitude or magnitude of current inhibition by AA was observed between $cPLA_2^{+/+}$ versus $cPLA_2^{-/-}$ neurons, indicating normal VGCC activity in $cPLA_2^{-/-}$ neurons. In contrast to VGCCs, no change in M-current inhibition occurred in $cPLA_2^{-/-}$ SCG neurons, demonstrating

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that breakdown of PIP_2 by PLC still must occur under these experimental conditions despite loss of L- and N-current inhibition.

Taken together the data indicate that L- and N-current inhibition occurs by a signal transduction cascade diverging from that mediating M-current inhibition [49,127,128,132,133]. This conclusion is supported by recent work using palmitoylated charged peptides to sequester PIP_2. Dialyzing low peptide concentrations into neurons disrupted M-current but not N-current modulation by M_1Rs [148]. Free fatty acids, most likely AA, liberated from the *sn*-2 position during phospholipid metabolism mediate current inhibition. AA itself, rather than a metabolite, serves as the effector since antagonizing cyclo-oxygenases, lipoxygenases or P-450 epoxygenases individually or all together had no effect on AA or Oxo-M's ability to inhibit current [23,39,49,133].

7.2 PLC, $cPLA_2$, and DAG lipase participate in M_1R modulation of Ca^{2+} currents

Recent findings help advance our understanding in how PIP_2 and AA regulate VGCC activity. In addition to PIP_2 breakdown by PLC and AA release by acutely activated $cPLA_2$, a third lipase called diacylglycerol (DAG) lipase appears to antagonize inhibition of native and recombinant L- and N-current (Fig. 5D) following M_1R stimulation [149]. DAG lipase α and β cleave fatty acids (normally stearic acid) preferentially from the *sn*-1 position of PIP_2 or from DAG [150,151]. This conclusion of DAG lipase involvement in the slow pathway is based on findings that pharmacologically antagonizing DAG lipases with the selective compound RHC-80267 minimizes L- and N-current inhibition. In contrast RHC-80267 has no effect on M-current inhibition by the slow pathway, N-current inhibition by direct G-protein binding to $Ca_V2.2$, or L-and N-current inhibition by exogenous AA [134], indicating its actions selectively target slow pathway modulation of L- and N-currents [108,149].

That three lipases are required for VGCC inhibition may seem implausible and excessively complicated. However, the need for all three lipases suggests a linear signal transduction cascade that generates a signaling molecule, such as AA, may not be responsible for Ca^{2+} current inhibition [127]. Similarly, the PIP_2 model where removal of the phosphoinositol head group by PLC is necessary and sufficient for observing Ca^{2+} current inhibition [104,106] also cannot explain why $cPLA_2^{-/-}$ neurons exhibit little L- and N-current inhibition, while M-current inhibition proceeds normally, indicating that PLC is active [127,134]. Thus both models fail in explaining all the results; however a remarkably simple "PIP_2-AA" model shown in Figure 5B successfully combines the essence of each model to explain slow pathway modulation of Ca^{2+} currents.

Moreover, the PIP_2-AA model successfully incorporates virtually all previously published findings from a number of research groups into an unexpectedly simple scheme. Oxo-M binds to M_1Rs which couple to G_q to activate PLC. Activated PLC directly cleaves the inositol head group in the *sn*-3 from PIP_2 molecules associated with channels Additionally PLC stimulates phosphorylation of $cPLA_2$. Activated $cPLA_2$ and DAG lipase liberate the two fatty acid tails of PIP_2 in situ by acting specifically at the *sn-2* and *sn-1* positions respectively of the glycerol backbone. Consequently, the released IP_3 and glycerol will enter the cytoplasm while the two freed fatty acids remain bound to channels. Alternatively the phosphoinositide headgroup could remain bound to the channel. Disassembling PIP_2 may uncouple a hydrophobic region of the channel that binds the fatty acid tails from the predicted charged pocket that interacts with the phosphoinositol head group. This separation may impede coordinated conformational changes that closed channels undergo as they reconfigure into an open conformation, thus changing the availability of channels to open. The bound free fatty acid tails antagonize binding of a PIP_2 to the site. Thus it is the loss of the glycerol backbone uncoupling the fatty acid tails from the head group that causes a decrease in channel opening, stabilizing a closed conformation. Three findings hint that a similar mechanism may occur for some K^+ channels: 1) the antagonistic

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actions of PIP_2 and AA on certain K^+ channels [78,152]; 2) overlapping binding sites for PIP_2 and AA at the proximal end of the C-terminus of Kir3 channels [78]; and 3) the importance of the head group and fatty acid tails for coupling voltage sensing to opening [153,154].

Another way to think of the consequences of PIP_2 breakdown may be that somehow voltage sensing uncouples from channel opening. Though sheer speculation, this idea brings to mind, intriguing crystal data from the MacKinnon lab of phospholipid placement in the inner regions of a voltage-dependent K^+ channel where just one phospholipid crystallized with each subunit [154]. The lipid tails sandwich between the voltage sensor and the inner pore with the head group pointing towards the cytoplasm. One could image that the phospholipid acts like Velcro between the two regions perhaps slipping as S6 flexes during opening only to re-attach as S6 straightens on closing. Whether VGCCs have a phospholipid in a similar region remains to be determined though a similar placement of PIP_2 in a VGCC would be an ideal site to explain PIP_2 and AA's actions at the "S" site. Few clues exist that hint as to whether the PIP_2 "R" site (which we hypothesizes confers enhancement by AA) is also located at the inner pore. It is possible that 4 phospholipids wedge between each voltage sensor and inner pore helix. Whether one or more of these wedged PIP_2s may confer reluctant gating kinetics is an attractive possibility. Unlike the crystallized K^+ channels, VGCCs do not have four-fold symmetry, so that homologous PIP_2 interaction sites may influence channel opening differently. Alternatively recent findings with K^+ channel structures reveal the importance of S1 for exerting the force on the outer pore region by the voltage sensor paddle [155]. Whether lipid packing in the crevices around S1 and the pore is important for channel opening no doubt will be an important question to answer.

Oxo-M also enhances N-current in SCG neurons similar to AA by increasing the voltage-sensitivity and kinetics of activation. Moreover muscarinic enhancement of N-current also involves PLA_2 activity [49]. In recombinant studies M_1Rs stimulation, as with exogenously applied AA, resulted in sustained enhancement of N-current only when $Ca_V2.2$ was expressed with the palmitoylated β_{2a} [102]. We imagine that by forming multiple binding sites with cytoplasmic domains of $Ca_V2.2$, the $Ca_V\beta2a$ protein effectively "docks" its two palmitic acids at a site extremely close to $Ca_V2.2$ promoting their interaction with the channel. This constraint creates a high local concentration of palmitic acid that promotes competition with AA and possibly PIP_2 for the "S" site acting there as a phospholipid mimic. No change in channel availability is observed with muscarinic stimulation, only the "R" site undergoes modulation. Thus the PIP_2-AA model successfully incorporates the actions of *three* lipid moities: PIP_2, AA and palmitoylated β2a. No doubt, lipid regulation of VGCCs does not occur exactly this way. This thinking however, creates a starting point for testing this molecular image of lipids competing for sites on VGCCs that effect channel opening. Critical to developing this model will be further experiments that examine whether mutating certain channel residues change the response to AA and to palmitoylation.

7.3 Control of PIP_2 interaction with VGCCs by three lipases provides a highly regulated mechanism for modulation

The PIP_2-AA model is appealing in that it provides more regulatory control of lipid interaction with channels compared to a "naked" channel with no bound lipid (Fig. 4). The local breakdown of PIP_2 should give rise to variety of byproducts that each alters VGCC gating in unique ways. Moreover, this new model resolves previous conceptual conflicts in mechanism and provides a framework for raising new predictions and pursuing a new direction of questioning around how lipids regulate VGCCs in normal and pathological situations. First, this model predicts that the lipid tails of PIP_2 and free AA interact with $Ca_V\alpha_1$ though whether PIP_2 or free AA act by directly interacting with the channel remains uncertain. The PIP_2-AA model is compatible with the idea that loss of PIP_2 from channels occurs following M_1R stimulation

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[104,106]. If we assume that PIP_2 directly interacts with the channel, then the simplest model would predict that $cPLA_2$ liberates AA on location and exogenously applied AA may dislodge PIP_2 by competing with its fatty acid tails for binding sites. Metabolizing PIP_2 still bound to channels can account for why inhibiting either PLA_2 or DAG lipase will antagonize channel modulation. However whether these enzymes are able to access PIP_2 molecules while bound to the channel needs to be tested more directly.

Though increases in free AA in the bath solution are detected following muscarinic stimulation [127], this new model predicts that free AA generated locally confers inhibition. In support of this idea, AA inhibits recombinant T-current, exhibiting similar inhibition as L- and N-currents [20,21]. Since T-current arises from channels with no accessory subunits, AA's actions are predicted to occur on the pore-forming subunit. Whether the Hill coefficient of ~2 for AA binding to T-channels [21] suggests that AA acts at two sites on the channel or that the two tails of PIP_2 act together at one site remains to be tested. This question of whether two tails participate in current inhibition raise an additional question of whether two AAs mediate inhibition or whether inhibition occurs from AA and the fatty acid, most likely stearic acid, from the *sn*-1 position; or just AA from the *sn*-2 position. The actions of stearic acid have not yet been tested on L- or N-current though other saturated fatty acids can enhance N-current (Barrett et al., 2001). Once liberated, AA may diffuse a short distance to its site of action. Whether other G_qPCRs require both $cPLA_2$ and DAG lipase to observe VGCC modulation has not been examined. Notably M_3Rs couple to G_q to activate an endogenous AA-dependent noncapacitative Ca^{2+} current in HEK cells that also requires $cPLA_2$ [156]. However, other G_qPCRs require both PLA_2 and DAGL activity and AA release to regulate a variety of cellular processes [156–163], documenting widespread association between DAGL and $cPLA_2$. These questions and many others arise from contemplating the predictions of this simple new model; the answers of which may be as surprising as what the field has discovered so far on how lipids regulate VGCC activity.

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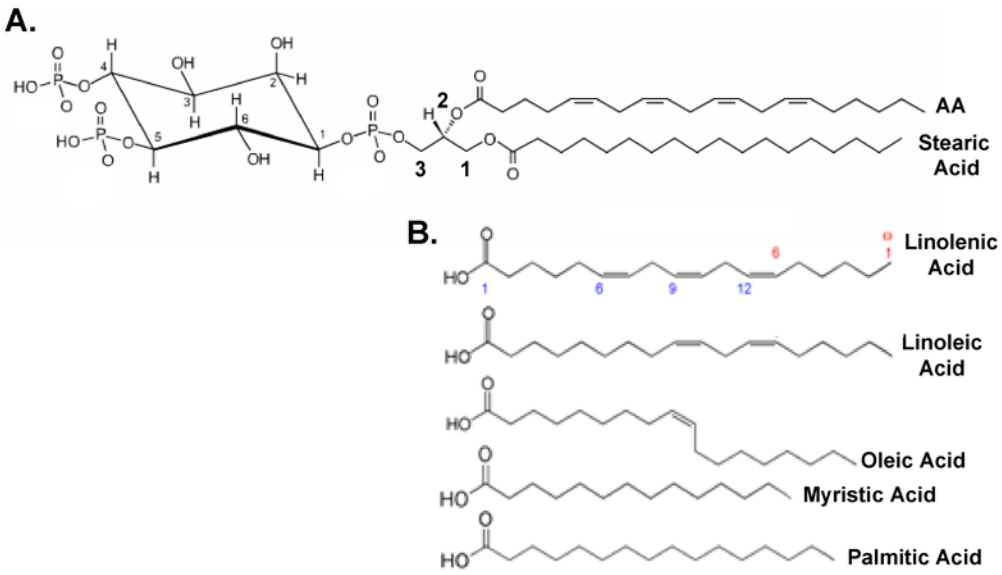

Figure 1.

PIP_2 is formed of a glycerol backbone with a phosphoinositol head group and two fatty acid tails. **A.** Stearic acid normally resides in the *sn*-1 position of PIP_2, while AA is found in the *sn*-2 position. The three carbons of the glycerol backbone are numbered in red. The inositol headgroup of PIP_2 attaches to the *sn*-3 carbon via a phosphoester linkage. The carbons of the inositol head group are numbered in black. Additional phosphate groups are located at carbons 4 and 5 of the inositol ring. **B.** Other fatty acids that can modulate VGCC activity. Carbon numbering starts from the carboxy end of fatty acids (shown in blue for linolenic acid) while defining the location of the first double-bond is determined from the amino terminus (shown in red).

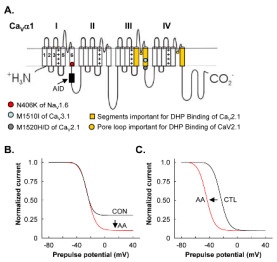

Figure 2.

Schematics of AA's effects on holding potential-dependent inactivation of Ca^{2+} channels. **A.** Topological orientation of a VGCC pore-forming subunit. The subunit is formed by four repeating domains (I–IV) each made up of six transmembrane segments (S1–S6) with each S4 containing at least four charged residues (+) that participate in voltage-sensing. Arrow indicates location of a high affinity binding site, called the alpha interaction domain (AID), for the β-subunit. Colored symbols approximate the location of critical residues that alter lipid regulation of identified voltage-gated ion channels. Yellow highlights identify regions critical for dihydropyridine (DHP) binding. **B.** In SCG neurons, AA (5 μM) increases the amount of inactivation as the holding potential becomes more positive. No shift in the voltage-sensitivity of inactivation is observed. **C.** For certain L- and T-channels, AA increases the voltage-sensitivity of inactivation, causing a leftward shift in the inactivation curve.

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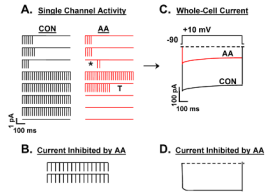

Figure 3.

Schematic illustrating AA's inhibitory actions on N-channel gating in SCG neurons. **A.** AA shifts the pattern of N-channel gating elicited with 750 ms test pulses. Eight idealized sweeps in the absence (CON) and presence of AA illustrate how AA shifts the pattern of unitary N-channel gating. Two minutes after puffing on micromolar concentrations of AA (red), the frequency of noninactivating sweeps decreases compared to control (CON), whereas of the numbers of null sweeps increase and are found clustered together. Additionally, in some sweeps, first latency increases (*) and some end with a long truncated closing (T). **B.** Noninactivating unitary N-channel activity is lost following AA. **C.** Idealized whole-cell current traces before (CON), and 2 min after puffing on AA. **D.** The whole-cell current inhibited by AA shows no fast inactivation consistent with a loss of unitary channel activity that is noninactivating (after Roberts-Crowley & Rittenhouse, 2008).

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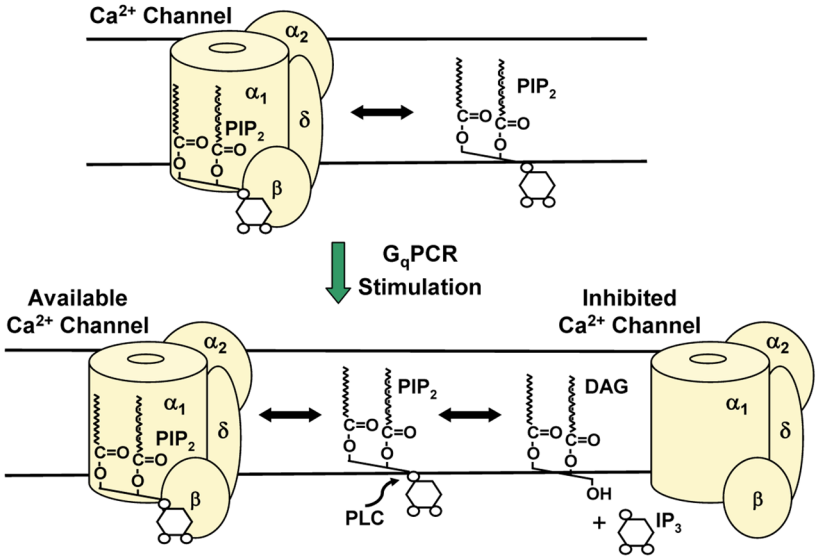

Figure 4.

The PIP_2 model of VGCC inhibition by G_qPCRs. Top; PIP_2 exists in a steady-state relationship with VGCCs, where it binds and unbinds with particular rate constants for each VGCC. When PLC is activated, it will metabolize PIP_2 that has dissociated from VGCCs.

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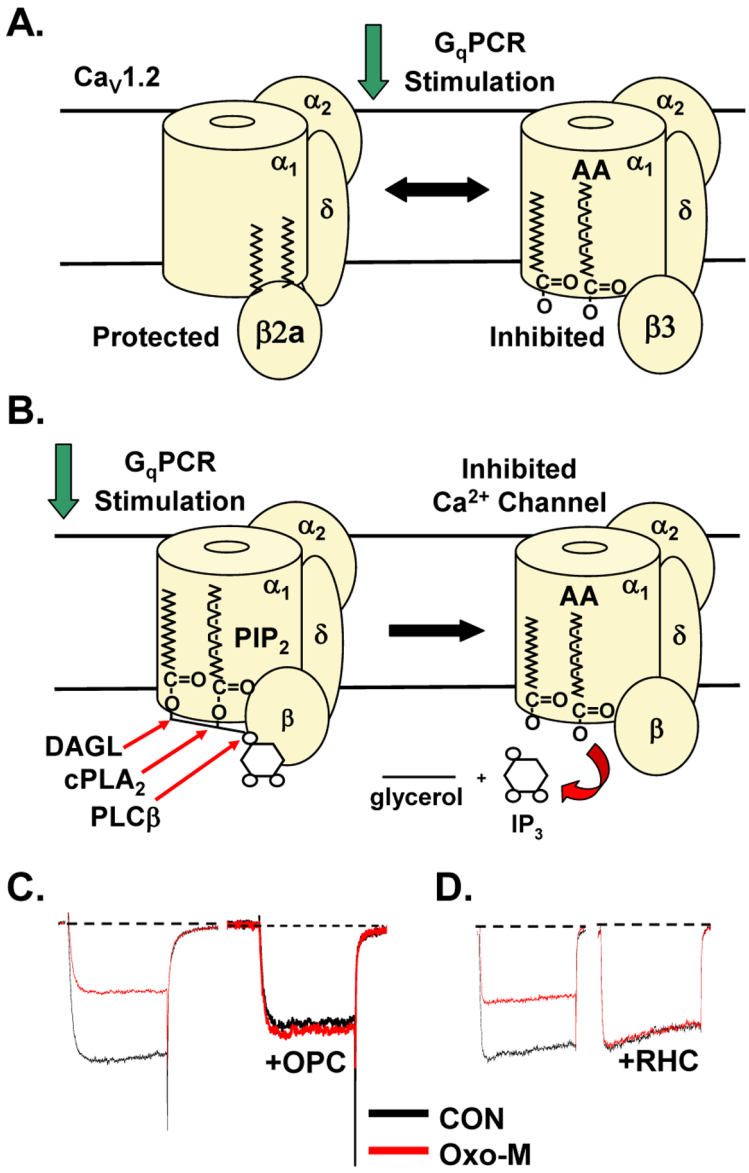

Figure 5.

PIP_2–AA model of VGCC inhibition by G_qPCRs. **A.** Model comparing $Ca_V1.3$ channels with palmitoylated β2a to inhibited channels with β3. **B.** PIP_2–AA model proposes that M_1R coupling to G_q activates PLC to remove the inositol head group from PIP_2 associated with the channel. Both DAG lipase (DAGL) and $cPLA_2$ must cleave the two fatty acid tails from the remaining glycerol backbone in order to observe current inhibition. Once freed from the glycerol backbone the two fatty acids confer inhibition. **C.** VGCC current modulation by 10 μM Oxo-M of a cortical neuron in the absence (left) or presence of the PLA_2 antagonist OPC (right). **D.** VGCC current modulation of a SCG neuron in the absence (left) or presence of the DAG lipase antagonist RHC 80267 (right).