Ion channel regulation by protein S-acylation

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Protein S-acylation, the reversible covalent fatty-acid modification of cysteine residues, has emerged as a dynamic posttranslational modification (PTM) that controls the diversity, life cycle, and physiological function of numerous ligand- and voltage-gated ion channels. S-acylation is enzymatically mediated by a diverse family of acyltransferases (zDHHCs) and is reversed by acylthioesterases. However, for most ion channels, the dynamics and subcellular localization at which S-acylation and deacylation cycles occur are not known. S-acylation can control the two fundamental determinants of ion channel function: (1) the number of channels resident in a membrane and (2) the activity of the channel at the membrane. It controls the former by regulating channel trafficking and the latter by controlling channel kinetics and modulation by other PTMs. Ion channel function may be modulated by S-acylation of both pore-forming and regulatory subunits as well as through control of adapter, signaling, and scaffolding proteins in ion channel complexes. Importantly, cross-talk of S-acylation with other PTMs of both cysteine residues by themselves and neighboring sites of phosphorylation is an emerging concept in the control of ion channel physiology. In this review, I discuss the fundamentals of protein S-acylation and the tools available to investigate ion channel S-acylation. The mechanisms and role of S-acylation in controlling diverse stages of the ion channel life cycle and its effect on ion channel function are highlighted. Finally, I discuss future goals and challenges for the field to understand both the mechanistic basis for S-acylation control of ion channels and the functional consequence and implications for understanding the physiological function of ion channel S-acylation in health and disease.

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Ion channels are modified by the attachment to the channel protein of a wide array of small signaling molecules. These include phosphate groups (phosphorylation), ubiquitin (ubiquitination), small ubiquitin-like modifier (SUMO) proteins (SUMOylation), and various lipids (lipidation). Such PTMs are critical for controlling the physiological function of ion channels through regulation of the number of ion channels resident in the (plasma) membrane; their activity, kinetics, and modulation by other PTMs; or their interaction with other proteins. S-acylation is one of a group of covalent lipid modifications (Resh, 2013). However, unlike N-myristoylation and prenylation (which includes farnesylation and geranylgeranylation), S-acylation is reversible (Fig. 1). Because of the labile thioester bond, S-acylation thus represents a dynamic lipid modification to spatiotemporally control protein function. The most common form of S-acylation, the attachment of the C16 lipid palmitate to proteins (referred to as S-palmitoylation), was first described more than 30 years ago in the transmembrane glycoprotein of the vesicular stomatitis virus and various mammalian membrane proteins (Schmidt and Schlesinger, 1979; Schlesinger et al., 1980). A decade later, S-acylated ion channels—rodent voltage-gated sodium channels (Schmidt and Catterall, 1987) and the M2 ion channel from the influenza virus (Sugrue et al., 1990)—were first characterized. Since then, more than 50 distinct ion channel subunits have been experimentally demonstrated to be S-acylated (Tables 1–3) as have a wide array of structural, signaling, and scaffolding proteins (for reviews see El-Husseini and Bredt, 2002; Linder and Deschenes, 2007; Fukata and Fukata, 2010; Greaves and Chamberlain, 2011; Resh, 2012). In the last few years, with the cloning of enzymes controlling S-acylation and development of various proteomic tools, we have begun to gain substantial mechanistic and physiological insight into how S-acylation may control multiple facets of the life cycle of ion channels: from their assembly, through their trafficking and regulation at the plasma membrane, to their final degradation (Fig. 2).

Here, I provide a primer on the fundamentals of S-acylation, in the context of ion channel regulation, along with a brief overview of tools available to interrogate ion channel S-acylation. I will discuss key examples of how S-acylation controls distinct stages of the ion channel life cycle before highlighting some of the key challenges for the field in the future.

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Abbreviations used in this paper: ABE, acyl-biotin exchange; acyl-CoA, acyl coenzyme A; AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; AQP4, aquaporin 4; $GABA_A$, γ-aminobutyric; LYPLA1, lysophospholipase 1; LYPLA2, lysophospholipase 2; nAChR, nicotinic acetylcholine receptor; P2X7, P2x purinoceptor 7; PDZ, PSD-95, Discs large, and ZO-1; PPT1, palmitoyl protein thioesterase; PSD-95, postsynaptic density protein 95; R7-BP, R7 binding protein; RAS, rat sarcoma; RyR1, skeletal muscle ryanodine receptor/Ca^{2+}-release channel; siRNA, small interfering RNA; STREX, stress-regulated exon; zDHHC, zinc finger–containing acyltransferase.

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Fundamentals of S-acylation: The what, when, where, and how

S-acylation: A fatty modification that controls multiple aspects of protein function. Protein S-acylation results from the attachment of a fatty acid to intracellular cysteine residues of proteins via a labile, thioester linkage (Fig. 1, A and B). Because the thioester bond is subject to nucleophilic attack, S-acylation, unlike other lipid modifications such as N-myristoylation and prenylation, is reversible. However, for most ion channels, as for other S-acylated proteins, the dynamics of S-acylation are poorly understood. Distinct classes of proteins can undergo cycles of acylation and deacylation that are very rapid (e.g., on the timescale of seconds, as exemplified by rat sarcoma [RAS] proteins), much longer (hours), or essentially irreversible during the lifespan of the protein (El-Husseini and Bredt, 2002; Linder and Deschenes, 2007; Zeidman et al., 2009; Fukata and Fukata, 2010; Greaves and Chamberlain, 2011; Resh, 2012). For most ion channels, in fact most S-acylated proteins, the identity of the native lipid species attached to specific cysteine residues is also largely unknown. However, the saturated C16:0 lipid palmitate is commonly thought to be the major lipid species in many S-acylated proteins (Fig. 1). Indeed, much of the earliest work on S-acylation involved the metabolic labeling of proteins in cells with tritiated [^{3}H]palmitate, an approach that still remains useful and important. However, lipids with different chain lengths and degrees of unsaturation (such as oleic and stearic acids) can also be added to cysteines via a thioester linkage, potentially allowing differential control of protein properties through the attachment of distinct fatty acids (El-Husseini and Bredt, 2002; Linder and Deschenes, 2007; Zeidman et al., 2009; Fukata

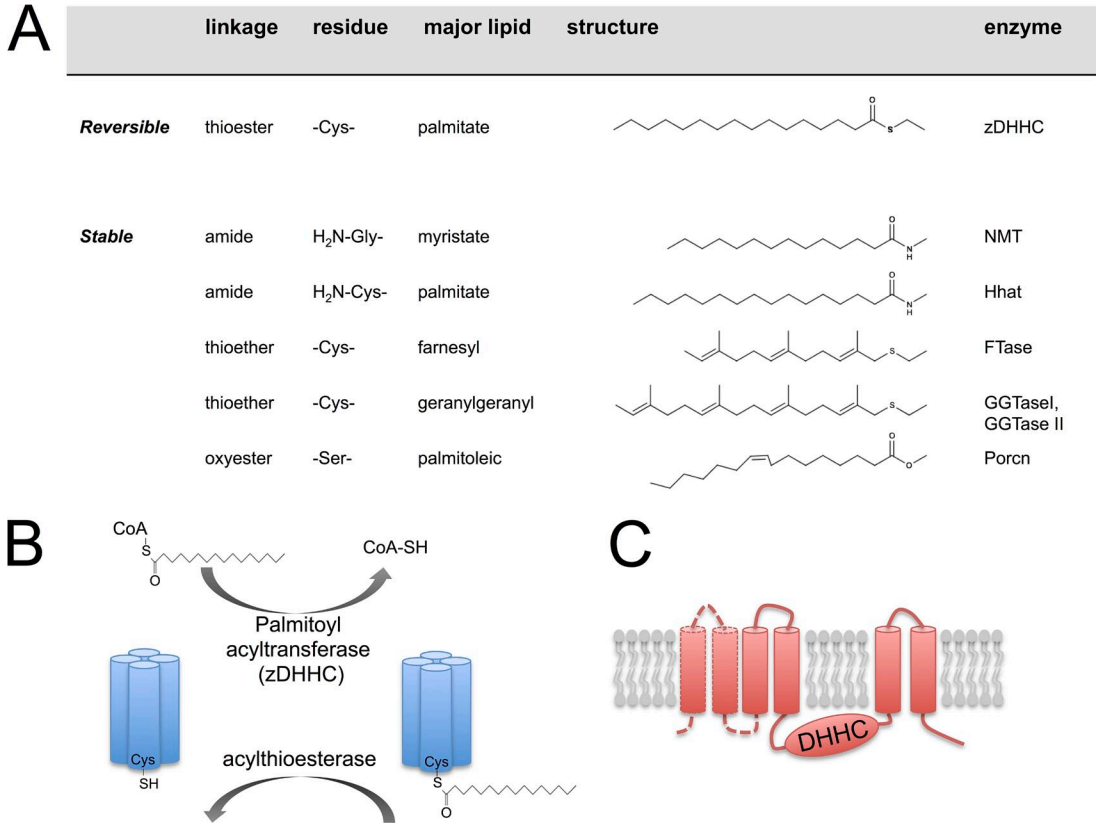

Figure 1. Protein S-acylation: a reversible lipid posttranslational modification of proteins. (A) Major lipid modifications of proteins. S-acylation is reversible due to the labile thioester bond between the lipid (typically, but not exclusively, palmitate) and the cysteine amino acid of is target protein. Other lipid modifications result from stable bond formation between either the N-terminal amino acid (amide) or the amino acid side chain in the protein (thioether and oxyester). The zDHHC family of palmitoyl acyltransferases mediates S-acylation with other enzyme families controlling other lipid modifications: N-methyltransferase (NMT) controls myristoylation of many proteins such as the src family kinase, Fyn kinase; and amide-linked palmitoylation of the secreted sonic hedgehog protein is mediated by Hedgehog acyltransferase (Hhat), a membrane-bound O-acyl transferase (MBOAT) family. Prenyl transferases catalyze farnesyl (farnesyltransferase, FTase) or geranylgeranyl (geranylgeranyl transferase I [GGTase I] and geranylgeranyl transferase II [GGTase II]) in small GTPase proteins such as RAS and the Rab proteins, respectively. Porcupine (Porcn) is a member of the MBOAT family acylates secreted proteins such as Wnt. (B) zDHHC enzymes typically use coenzyme A (CoA)-palmitate; however, other long chain fatty acids (either saturated or desaturated) can also be used. Deacylation is mediated by several acylthioesterases of the serine hydrolase family. (C) zDHHC acyltransferases (23 in humans) are predicted transmembrane proteins (typically with 4 or 6 transmembrane domains) with the catalytic DHHC domain located in a cytosolic loop.

TABLE 1

Pore-forming subunits of ion channels experimentally determined to be S-acylated

Channel	Subunit	Gene	Candidate S-acylation sites	UniProt ID	References
Ligand-gated					
AMPA	GluA1	*Gria1*	[593]FSLGAFMQQGCDISPRSLSGRI	P23818	Hayashi et al., 2005
			[819]LAMLVALIEFCYKSRSESKRMK	P23818	Hayashi et al., 2005
	GluA2	*Gria2*	[600]FSLGAFMRQGCDISPRSLSGRI	P23819	Hayashi et al., 2005
			[826]LAMLVALIEFCYKSRAEAKRMK	P23819	Hayashi et al., 2005
	GluA3	*Gria3*	[605]FSLGAFMQQGCDISPRSLSGRI	Q9Z2W9	Hayashi et al., 2005
			[831]LAMMVALIEFCYKSRAESKRMK	Q9Z2W9	Hayashi et al., 2005
	GluA4	*Gria4*	[601]FSLGAFMQQGCDISPRSLSGRI	Q9Z2W8	Hayashi et al., 2005
			[827]LAMLVALIEFCYKSRAEAKRMK	Q9Z2W8	Hayashi et al., 2005
$GABA_A$	γ2	*Gabrg2*	[405]QERDEEYGYECLDGKDCASFFCCFEDCRTGAWRHGRI	P22723	Rathenberg et al., 2004; Fang et al., 2006
Kainate	GluK2	*Grik2*	[848]KNAQLEKRSFCSAMVEELRMSLKCQRRLKHKPQAPV	P39087	Pickering et al., 1995
nAChR	α4	*Chrna4*	[263]TVLVFYLPSECGEKVTLCISV	O70174	Alexander et al., 2010; Amici et al., 2012
	α7	*Chrna7*	ND		Alexander et al., 2010; Drisdel et al., 2004
	β2	*Chrnb2*	ND		Alexander et al., 2010
NMDA	GluN2A	*Grin2a*	[838]EHLFYWKLRFCFTGVCSDRPGLLFSISRGIYSCIHGVHIEEKK	P35436	Hayashi et al., 2009
			[1204]SDRYRQNSTHCRSCLSNLPTYSGHFTMRSPFKCDACLRMGNLYDID	P35436	Hayashi et al., 2009
	GluN2B	*Grin2b*	[839]EHLFYWQFRHCFMGVCSGKPGMVFSISRGIYSCIHGVAIEERQ	Q01097	Hayashi et al., 2009
			[1205]DWEDRSGGNFCRSCPSKLHNYSSTVAGQNSGRQACIRCEACKKAGNLYDIS	Q01097	Hayashi et al., 2009
P2X7	P2X7	*P2rx7*	[361]AFCRSGVYPYCKCCEPCTVNEYYYRKK	Q9Z1M0	Gonnord et al., 2009
			[469]APKSGDSPSWCQCGNCLPSRLPEQRR	Q9Z1M0	Gonnord et al., 2009
			[488]PEQRRALEELCCRRKPGRCITT	Q9Z1M0	Gonnord et al., 2009
			[562]DMADFAILPSCCRWRIRKEFPK	Q9Z1M0	Gonnord et al., 2009
Voltage gated					
Potassium					
BK, maxiK	KCa1.1	*Kcnma1*	[43]WRTLKYLWTVCCHCGGKTKEAQKI	Q08460	Jeffries et al., 2010
			[635]MSIYKRMRRACCFDCGRSERDCSCM	Q08460	Tian et al., 2008; 2010
	Kv1.1	*Kcna1*	[233]SFELVVRFFACPSKTDFFKNI	P16388	Gubitosi-Klug et al., 2005
	Kv1.5	*Kcna5*	[16]LRGGGEAGASCVQSPRGECGC	Q61762	Jindal et al., 2008
			[583]VDLRRSLYALCLDTSRETDL-stop	Q61762	Zhang et al., 2007; Jindal et al., 2008
Sodium	NaV1.2	*Scn2a1*	ND		Schmidt and Catterall, 1987
			[640]MNGKMHSAVDCNGVVSLVGGP	P04775	Bosmans et al., 2011
			[1042]LEDLNNKKDSCISNHTTIEIG	P04775	Bosmans et al., 2011
			[1172]TEDCVRKFKCCQISIEEGKGK	P04775	Bosmans et al., 2011
Other channels					
Aquaporin	AQP4	*Aqp4*	[3]DRAAARRWGKCGHSCSRESIMVAFK	P55088	Crane and Verkman, 2009; Suzuki et al., 2008
CFTR	CFTR	*CFTR*	[514]EYRYRSVIKACQLEEDISKFAEKD	P13569	McClure et al., 2012
			[1385]RRTLKQAFADCTVILCEHRIEA	P13569	McClure et al., 2012
Connexin	Cx32	*Gjb1*	[270]GAGLAEKSDRCSAC-stop	P28230	Locke et al., 2006
ENaC	ENaC β	*Scnn1b*	[33]TNTHGPKRIICEGPKKKAMWFL	Q9WU38	Mueller et al., 2010
			[547]WITIIKLVASCKGLRRRRPQAPY	Q9WU38	Mueller et al., 2010
	ENaC γ	*Scnn1g*	[23]PTIKDLMHWYCLNTNTHGCRRIVVSRGRL	Q9WU39	Mukherjee et al., 2014
Influenza M2	M2	—	[40]LWILDRLFFKCIYRFFEHGLK	Q20MD5	Sugrue et al., 1990; Holsinger et al., 1995; Veit et al., 1991
RyR1	RYR1	*Ryr1*	[14]LRTDDEVVLQCSATVLKEQLKLCLAAEGFGNRL	P11716	Chaube et al., 2014
			[110]RHAHSRMYLSCLTTSRSMTDK	P11716	Chaube et al., 2014
			[243]RLVYYEGGAVCTHARSLWRLE	P11716	Chaube et al., 2014
			[295]EDQGLVVVDACKAHTKATSFC	P11716	Chaube et al., 2014
			[527]ASLIRGNRANCALFSTNLDWV	P11716	Chaube et al., 2014
			[1030]ATKRSNRDSLCQAVRTLLGYG	P11716	Chaube et al., 2014
			[1664]SHTLRLYRAVCALGNNRVAHA	P11716	Chaube et al., 2014

TABLE 1 *(Continued)*

Channel	Subunit	Gene	Candidate S-acylation sites	UniProt ID	References
			[2011]HFKDEADEED**C**PLPEDIRQDL	P11716	Chaube et al., 2014
			[2227]KMVTSCCRFL**C**YFCRISRQNQ	P11716	Chaube et al., 2014
			[2316]KGYPDIGWNP**C**GGERYLDFLR	P11716	Chaube et al., 2014
			[2353]VVRLLIRKPE**C**FGPALRGEGG	P11716	Chaube et al., 2014
			[2545]EMALALNRYL**C**LAVLPLITK**C**APLFAGTEHR	P11716	Chaube et al., 2014
			[3160]DVQVSCYRTL**C**SIYSLGTTKNTYVEKLRPALGE**C**LARLAAAMPV	P11716	Chaube et al., 2014
			[3392]LLVRDEFSVL**C**RDLYALYPLL	P11716	Chaube et al., 2014
			[3625]SKQRRRAVVA**C**FRMTPLYNLP	P11716	Chaube et al., 2014

Common channel abbreviation and subunit as well as gene names are given. Candidate S-acylation sites: experimentally determined cysteine residues (bold) with flanking 10 amino acids. Underlines indicate predicted transmembrane domains. Amino acid numbering corresponds to the UniProt ID. References: selected original supporting citations.

and Fukata, 2010; Greaves and Chamberlain, 2011; Resh, 2012).

S-acylation increases protein hydrophobicity and has thus been implicated in controlling protein function in many different ways. Most commonly, as with membrane-associated proteins like RAS and postsynaptic density protein 95 (PSD-95), S-acylation controls membrane attachment and intracellular trafficking. However, S-acylation can also control protein–protein interactions, protein targeting to membrane subdomains, protein stability, and regulation by other PTMs such as phosphorylation (El-Husseini and Bredt, 2002; Fukata and Fukata, 2010; Linder and Deschenes, 2007; Greaves and Chamberlain, 2011; Shipston, 2011; Resh, 2012). Evidence for all these mechanisms in controlling ion channel function is beginning to emerge.

Enzymatic control of S-acylation by zinc finger–containing acyltransferase (zDHHC) transmembrane acyltransferases. Although autoacylation of some proteins has been reported in the presence of acyl coenzyme A (acyl-CoA; Linder and Deschenes, 2007), most cellular S-acylation, in organisms from yeast to humans, is thought to be enzymatically driven by a family of protein acyltransferases (gene family: zDHHC, with ∼23 members in mammals). These acyltransferases are predicted to be transmembrane zinc finger containing proteins (Fig. 1 C) that include a conserved Asp-His-His-Cys (DHHC) signature sequence within a cysteine-rich stretch of ∼50 amino acids critical for catalytic activity (Fukata et al., 2004). Although the enzymatic activity and lipid specificity of all of the zDHHC family proteins has not been elucidated, S-acylation is thought to proceed through a common, two step "ping pong" process (Mitchell et al., 2010; Jennings and Linder, 2012). However, different zDHHC enzymes may show different acyl-CoA substrate specificities. For example, zDHHC3 activity is reduced by acyl chains of >16 carbons (e.g., stearoyl CoA), whereas zDHHC2 efficiently transfers acyl chains of 14 carbons or longer (Jennings and Linder, 2012). The local availability of different acyl-CoA species may thus play an important role in differentially controlling protein S-acylation.

TABLE 2

Accessory subunits and selected ion channel adapter proteins

Channel	Subunit	Gene	Candidate S-acylation sites	UniProt ID	References
Voltage gated					
Calcium	CaVβ2a	*Cacnb2*	[1]MQ**CC**GLVHRRRVRV	Q8CC27	Chien et al., 1996; Stephens et al., 2000; Heneghan et al., 2009; Mitra-Ganguli et al., 2009
Potassium	KChip2	*Kcnip2*	[34]LKQRFLKLLP**CC**GPQALPSVSE	Q9JJ69	Takimoto et al., 2002
	KChip3	*Kcnip3*	[35]PRFTRQALMR**CC**LIKWILSSAA	Q9QXT8	Takimoto et al., 2002
	BK β4	*Kcnmb4*	[193]VGVLIVVLTI**C**AKSLAVKAEA	Q9JIN6	Chen et al., 2013
Adapter proteins that interact with ion channels	PICK1	*Pick1*	[404]TGPTDKGGSW**C**DS-stop	Q62083	Thomas et al., 2013
	Grip1b	*Grip1*	[1]MPGWKKNIPI**C**LQAEEQERE	Q925T6-2	Thomas et al., 2012; Yamazaki et al., 2001
	psd-95	*Dlg4*	[1]MD**C**L**C**IVTTKKYR	Q62108	Topinka and Bredt, 1998
	S-delphilin	*Grid2ip*	[1]MS**C**LGIFIPKKH	Q0QWG9-2	Matsuda et al., 2006
	Ankyrin-G	*Ank3*	[60]YIKNGVDVNI**C**NQNGLNALHL	F1LNM3	He et al., 2012

Common channel abbreviation and subunit as well as gene names are given. Candidate S-acylation sites: experimentally determined cysteine residues (bold) with flanking 10 amino acids. Underlines indicate predicted transmembrane domains. Amino acid numbering corresponds to the UniProt ID. References: selected original supporting citations.

We know very little about how zDHHC activity and function are regulated. Dimerization of zDHHCs 2 and 3 reduces their zDHHC activity compared with the monomeric form (Lai and Linder, 2013). Moreover, zDHHCs undergo autoacylation and contain predicted sites for other posttranslational modifications. Almost half of all mammalian zDHHCs contain a C-terminal PSD-95, Discs large, and ZO-1 (PDZ) domain binding motif, allowing them to assemble with various PDZ domain proteins that regulate ion channels (such as GRIP1b and PSD-95; Thomas and Hayashi, 2013). Other protein interaction domains are also observed in zDHHCs, such as ankyrin repeats in zDHHC17 and zDHHC13 (Greaves and Chamberlain, 2011). Indeed, increasing evidence suggests that various ion channels—including the ligand-gated γ-aminobutyric ($GABA_A$), α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), and NMDA receptors and the large conductance calcium- and voltage-activated (BK) potassium channels—can assemble in complexes with their cognate zDHHCs.

The expansion of the number of zDHHCs in mammals (23 vs. 7 in yeast), together with increased prevalence of PDZ interaction motifs, likely represents evolutionary gain-of-function mechanisms to diversify zDHHC function (Thomas and Hayashi, 2013). Evolutionary gain of function is also seen in ion channel subunit orthologues through acquisition of S-acylated cysteine residues absent in orthologues lower in the phylogenetic tree (such as the transmembrane domain 4 [TM4] sites in GluA1–4 subunits of AMPA receptors [Thomas and Hayashi, 2013] and the sites in the alternatively spliced stress-regulated exon [STREX] insert in the C terminus of the BK channel [Tian et al., 2008]). Importantly, some zDHHCs may have additional roles beyond their acyltransferase function. For example, the *Drosophila melanogaster* zDHHC23 orthologue lacks the catalytic DHHC sequence, and thus protein acyltransferase activity, and is a chaperone involved in protein trafficking (Johswich et al., 2009), whereas mammalian zDHHC 23 has a functional zDHHC motif and, in addition to S-acylating BK channels (Tian et al., 2012), can bind and regulate, but does not S-acylate, neuronal nitric oxide synthase (nNOS; Saitoh et al., 2004).

However, as with most S-acylated proteins, the identity of the zDHHCs that modify specific cysteine residues on individual ion channels is not known. Indeed, relatively few studies have tried to systematically identify the zDHHCs controlling ion channel function (Tian et al., 2010, 2012). Thus we are largely ignorant of the extent to which different zDHHCs may have specific ion channel targets or may display specificity. Some details are beginning to emerge: for example, zDHHC3 appears to be a rather promiscuous acyltransferase reported to S-acylate several ion channels (Keller et al., 2004; Hayashi et al., 2005, 2009; Tian et al., 2010), whereas distinct sites on the same ion channel subunit can be modified by distinct subsets of zDHHCs (Tian et al., 2010, 2012). Although we are still in the foothills of understanding the substrates and physiological roles of different zDHHCs, mutation or loss of function in zDHHCs is associated with an increasing number of human disorders, including cancers, various neurological disorders (such as Huntington's disease and X-linked mental retardations), and disruption of endocrine function in diabetes (Linder and Deschenes, 2007; Fukata and Fukata, 2010; Greaves and Chamberlain, 2011; Resh, 2012).

Deacylation is controlled by acylthioesterases. Protein deacylation is enzymatically driven by a family of acylthioesterases that belong to the serine hydrolase superfamily (Zeidman et al., 2009; Bachovchin et al., 2010). Indeed, using a broad spectrum serine lipase inhibitor, global proteomic S-acylation profiling identified a subset of serine hydrolases responsible for depalmitoylation (Martin et al., 2012). This study identified both the previously known acylthioesterases as well as potential novel candidate acylthioesterases. The acylthioesterases responsible

TABLE 3

Other channels identified in mammalian palmitoylome screens

Channel	Gene
Anion	
Chloride channel 6	*Clcn6*
Chloride intracellular channel 1	*Clic1*
Chloride intracellular channel 4	*Clic4*
Tweety homologue 1	*Ttyh1*
Tweety homologue 3	*Ttyh3*
Voltage-dependent anion channel 1	*Vdac1*
Voltage-dependent anion channel 2	*Vdac2*
Voltage-dependent anion channel 3	*Vdac3*
Calcium	
Voltage-dependent, L-type subunit α 1S	*Cacna1s*
Voltage-dependent, gamma subunit 8	*Cacng8*
Cation	
Amiloride-sensitive cation channel 2	*Accn2*
Glutamate	
Ionotropic, Δ1	*Grid1*
Perforin	
Perforin 1	*Prf1*
Potassium	
Voltage-gated channel, subfamily Q, member 2	*Kcnq2*
Sodium	
Voltage-gated, type I, α	*Scn1a*
Voltage-gated, type III, α	*Scn3a*
Voltage-gated, type IX, α	*Scn9a*
Transient receptor potential	
Cation channel, subfamily V, member 2	*Trpv2*
Cation channel, subfamily M, member 7	*Trpm7*

Channels identified in global S-acylation screens (Wan et al., 2007, 2013; Kang et al., 2008; Martin and Cravatt, 2009; Yang et al., 2010; Yount et al., 2010; Merrick et al., 2011; Wilson et al., 2011; Jones et al., 2012; Ren et al., 2013; Chaube et al., 2014) and not independently characterized as in Tables 1 and 2. Common channel abbreviation and gene names are given.

for deacylating ion channels, as for most other acylated membrane proteins, have not been clearly defined. Furthermore, the extent to which different members of the serine hydrolase superfamily display acylthioesterase activity toward ion channels is not known. Moreover, whether additional mechanisms of nucleophilic attack of the labile thioester bond may also mediate deacylation is not known.

Homeostatic control of deacylation of many signaling proteins is likely affected by a family of cytosolic acyl protein thioesterases including lysophospholipase 1 (LYPLA1; Yeh et al., 1999; Devedjiev et al., 2000) and lysophospholipase 2 (LYPLA2; Tomatis et al., 2010). These enzymes show some selectivity for different S-acylated peptides (Tomatis et al., 2010). Indeed, LYPLA1, but not LYPLA2, deacylates the S0-S1 loop of BK channels, leading to Golgi retention of the channel (Tian et al., 2012). A splice variant of the related LYPLAL1 acylthioesterases can also deacylate the BK channel S0-S1 loop, although the crystal structure of LYPLAL1 suggests it is likely to have a preference for lipids with shorter chains than palmitate (Bürger et al., 2012). Thus, whether lipid preference depends on protein interactions or if BK channels have multiple lipid species at the multicysteine S0-S1 site remain unknown. Relatively little is known about the regulation of these acylthioesterases; however, both LYPLA1 and LYPLA2 are themselves S-acylated. This controls their trafficking and association with membranes (Kong et al., 2013; Vartak et al., 2014) and may be important for accessing the thioesterase bond at the membrane interface. Additional mechanisms may promote accessibility of thioesterases to target cysteines. For example, the prolyl isomerase protein FKBP12 binds to palmitoylated RAS, and promotes RAS deacylation via a proline residue near the S-acylated cysteine (Ahearn et al., 2011).

Upon lysosomal degradation, many proteins are deacylated by the lysosomal palmitoyl protein thioesterase (PPT1; Verkruyse and Hofmann, 1996), and mutations in PPT1 lead to the devastating condition of infantile neuronal ceroid lipofuscinosis (Vesa et al., 1995; Sarkar et al., 2013). However, PPT1 can also be found in synaptic and other transport vesicles, and genetic deletion of PPT1 in mice may have different effects on similar proteins, which suggests roles beyond just lysosomal mediated degradation. For example, in PPT1 knockout mice the total expression and surface membrane abundance of the GluA4 AMPA receptor subunit was decreased, whereas PPT1 knockout had no effect on GluA1 or GluA2 AMPA subunits nor on NMDA receptor subunit expression or surface abundance (Finn et al., 2012).

However, for most ion channels, the questions of which enzymes control deacylation, where this occurs in cells, and how the time course of acylation–deacylation cycles are regulated are largely unknown. Thus, whether deacylation plays an active role in channel regulation remains poorly understood.

S-acylation occurs at membrane interfaces. Because the zDHHCs are transmembrane proteins and the catalytic DHHC domain is located at the cytosolic interface with membranes (Fig. 1 C), S-acylation of ion channels occurs at membrane interfaces. Although overexpression studies of recombinant mammalian zDHHCs in heterologous expression systems have indicated that most zDHHCs are localized to either the endoplasmic reticular or Golgi apparatus membranes (or both; Ohno et al., 2006), some zDHHCs are also found in other compartments, including the plasma membrane and trafficking endosomes (Thomas et al., 2012; Fukata et al., 2013). We know very little about the regulation and subcellular localization of most native zDHHC enzymes in different cell types, in large part because of the lack of high-quality antibodies that recognize native zDHHCs. However, some enzymes, including zDHHC2, can dynamically shuttle between different membrane compartments. Activity-dependent redistribution of zDHHC2 in neurons (Noritake et al., 2009) controls S-acylation of the postsynaptic scaffolding protein PSD-95, thereby regulating NMDA receptor function. Intriguingly, as ion channels themselves determine cellular excitability, this may provide a local feedback mechanism to regulate S-acylation status. Thus, although different zDHHCs may reside in multiple membrane compartments through which ion channels traffic, the subcellular location at which most ion channels are S-acylated, as well as the temporal dynamics, is largely unknown. As discussed below (see the "Tools to analyze ion channel S-acylation" section), we are starting to unravel some of the details, with ER exit, Golgi retention, recycling endosomes, and local plasma membrane compartments being key sites in the control of ion channel S-acylation (Fig. 2).

Local membrane and protein environment determines cysteine S-acylation. The efficiency of S-acylation of cysteine residues is likely enhanced by its localization at membranes because the local concentration of fatty acyl CoA is increased near hydrophobic environments (Bélanger et al., 2001). Furthermore, S-acylation of polytopic transmembrane proteins such as ion channels would be facilitated when S-acylated cysteines are bought into close proximity of membranes by membrane targeting mechanisms such as transmembrane helices (Figs. 3 and 4). However, the S-acylated cysteine is located within 10 amino acids of a transmembrane domain in only ∼20% of identified S-acylated ion channel subunits, such as the TM4 site of GluA1–4 (Tables 1 and 2). Most S-acylated cysteines are located either within intracellular loops (∼40%: Fig. 3, A and B) or the N- or C-terminal cytosolic domains (∼5% and 35%, respectively; Fig. 3, A and B). Furthermore, the majority of S-acylated cysteines located in intracellular loops or intracellular N- or C-terminal domains of ion channel subunits are within predicted regions of protein disorder (Fig. 3 B).

This suggests that S-acylation may provide a signal to promote conformational restraints on such domains, in particular by providing a membrane anchor. For these sites, additional initiating membrane association signals are likely required adjacent to the site of S-acylation. Likely candidates include other hydrophobic domains (as for the TM2 site in GluA1–4 subunits; Fig. 4 A) and other lipid anchors (e.g., myristoylation in src family kinases, such as Fyn kinase). However, in >30% of S-acylated ion channels, the S-acylated cysteine is juxtaposed to a (poly) basic region of amino acids that likely allows electrostatic interaction with negative membrane phospholipids. The BK channel pore-forming α subunit, encoded by the KCNMA1 gene, provides a clear example of this latter mechanism. This channel is S-acylated within an alternatively spliced domain (STREX) in its large intracellular C terminus (Fig. 4 C). Immediately upstream of the S-acylated dicysteine motif is a polybasic region enriched with arginine and lysine. Site-directed mutation of these basic amino acids disrupts S-acylation of the downstream cysteine residues (Jeffries et al., 2012). Furthermore, phosphorylation of a consensus PKA site (i.e., introduction of negatively charged phosphate) into the polybasic domain prevents STREX S-acylation. Thus, at the STREX domain, an electrostatic switch, controlled by phosphorylation, is an important determinant of BK channel S-acylation. In other proteins, cysteine reactivity is also enhanced by proximity to basic (or hydrophobic) residues (Bélanger et al., 2001; Britto et al., 2002; Kümmel et al., 2010). Furthermore, cysteine residues are subject to a range of modifications including nitrosylation, sulphydration, reduction-oxidation (REDOX) modification, and formation of disulphide bonds (Sen and Snyder, 2010). Evidence is beginning to emerge that these reversible modifications are mutually competitive for S-acylation of target cysteines (see the "S-acylation and posttranslational cross-talk controls channel trafficking and activity" section; Ho et al., 2011; Burgoyne et al., 2012).

Although these linear amino acid sequence features are likely to be important for efficient S-acylation, there is no canonical "consensus" S-acylation motif analogous to the linear amino acid sequences that predict sites of phosphorylation. Of the experimentally validated ion channel subunits shown to be S-acylated, ∼70% of candidate S-acylated cysteines are predominantly characterized as single cysteine (-C-) motifs, whereas dicysteine motifs (-CC-) and ($CX_{(1–3)}C$-) motifs comprise ∼10% and 20% of all sites, respectively (Table 1 and Fig. 3 B). However, several freely available online predictive tools have proved successful in characterizing potential new palmitoylation targets. In particular, the latest iteration of the multiplatform CSS-palm 4.0 tool (Ren et al., 2008) exploits a Group-based prediction algorithm by comparing the surrounding amino acid sequence similarity to that of a set of 583 experimentally determined

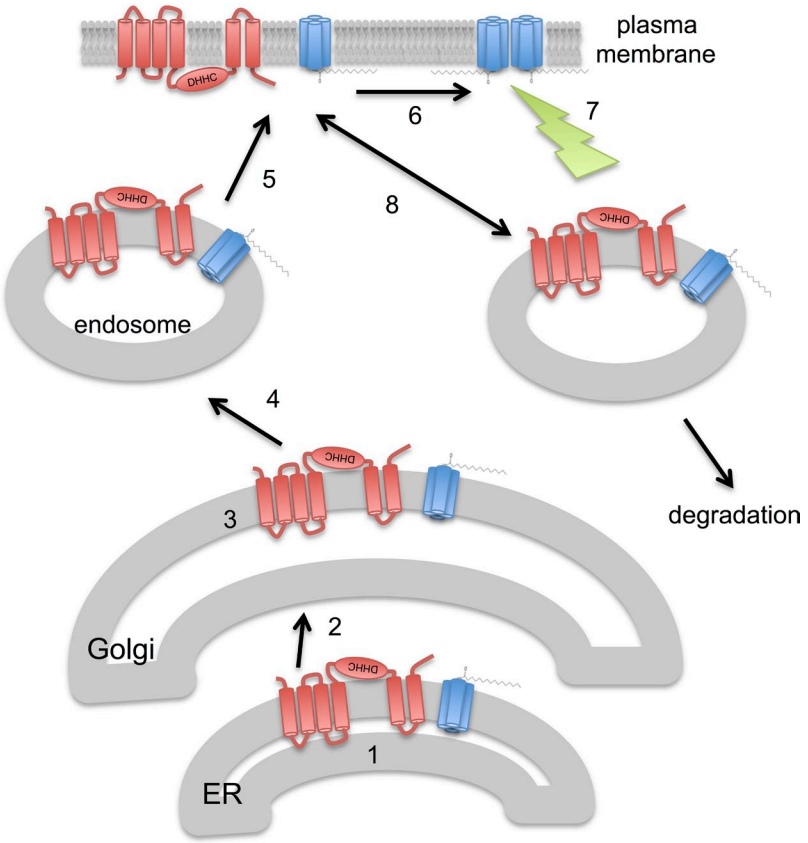

Figure 2. Protein S-acylation and regulation of the ion channel lifecycle zDHHCs are found in multiple membrane compartments and regulate multiple steps in the ion channel lifecycle including: (1) assembly and (2) ER exit; (3) maturation and Golgi exit; (4) sorting and trafficking; (5) trafficking and insertion into target membrane; (6) clustering and localization in membrane microdomains; control of properties, activity (7), and regulation by other signaling pathways; and (8) internalization, recycling, and final degradation.

S-acylation sites from 277 distinct proteins. CSS-palm 4.0 predicts >80% of the experimentally identified ion channel S-acylation sites (Tables 1–3) and suggests that >50% of human channel subunits may be S-acylated.

Location of S-acylated cysteine is important for differential control of channel function. Many proteins are S-acylated at multiple sites. A remarkable example of this, in the ion channel field, is the recent identification of 18 S-acylated cysteine residues in the skeletal muscle ryanodine receptor/Ca^{2+}-release channel (RyR1). The S-acylated cysteine residues are distributed throughout the cytosolic N terminus, including domains important for protein–protein interactions (Chaube et al., 2014). Although deacylation of skeletal muscle RyR1 reduces RyR1 activity, the question of which of these cysteine residues in RyR1 are important for this effect and whether distinct S-acylated cysteines in RyR1 control different functions and/or properties remains to be determined.

However, both ligand-gated (NMDA and AMPA) and voltage-gated (BK) channels provide remarkable insights into how S-acylation of different domains within the same polytopic protein can exert fundamentally distinct effects (Fig. 4). For example, S-acylation of the hydrophobic cytosolic TM2 domain located at the membrane interface of the AMPA GluA1 subunit (Fig. 4 A) decreases AMPA receptor surface expression by retaining the subunit at the Golgi apparatus (Hayashi et al., 2005). In contrast, depalmitoylation of the C-terminal cysteine in GluA1 results in enhanced PKC-dependent phosphorylation of neighboring serine residues, which results in increased interaction with the actin-binding protein 4.1N in neurons, leading to enhanced AMPA plasma membrane insertion (Lin et al., 2009). S-acylation of the C-terminal cluster of cysteine residues (Fig. 4 B, Cys II site) in GluN2A and GluN2B controls Golgi retention, whereas palmitoylation of the cysteine cluster (Cys I site) proximal to the M4 transmembrane domain controls channel internalization (Hayashi et al., 2009). Distinct roles of S-acylation on channel trafficking and regulation are also observed in BK channels (Figs. 4 C and 5). S-acylation of the N-terminal intracellular S0-S1 linker controls surface expression, in part by controlling ER and Golgi exit of the channel (Jeffries et al., 2010; Tian et al., 2012), whereas S-acylation of the large intracellular C terminus, within the alternatively spliced STREX domain, controls BK channel regulation by AGC family protein kinases (Tian et al., 2008; Zhou et al., 2012).

How does S-acylation of distinct domains control such behavior, and are distinct sites on the same protein acylated by distinct zDHHCs? A systematic small interfering RNA (siRNA) screen of zDHHC enzymes mediating BK channel S-acylation indicated that distinct subsets of zDHHCs modify discrete sites. The S0-S1 loop is S-acylated by zDHHCs 22 and 23, whereas the STREX domain is S-acylated by several zDHHCs including 3, 9, and 17 (Tian et al., 2008, 2012). In both cases, each domain has two distinct S-acylated cysteines; however, whether these cysteines are differentially S-acylated by specific zDHHCs is unknown, Furthermore, whether multiple zDHHCs are required because the domains undergo repeated cycles of S-acylation and deacylation, and thus

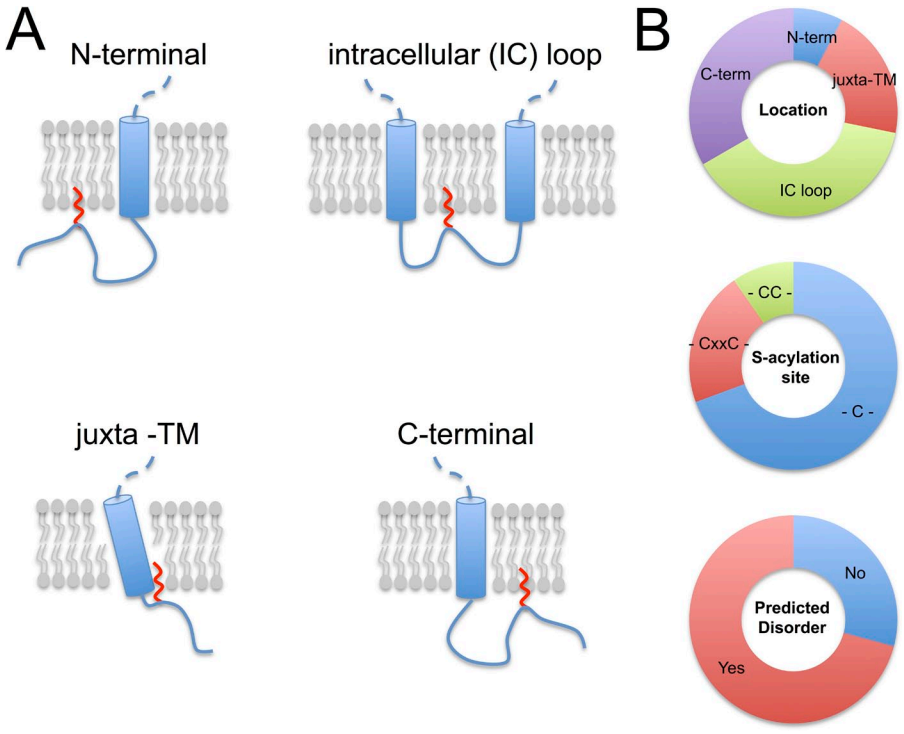

Figure 3. S-acylation sites in ion channel pore-forming subunits. (A) Schematic illustrating different locations of cysteine S-acylation in transmembrane ion channels subunits. (B) Relative proportion of identified S-acylated cysteine residues: in each location indicated in A (top); in -C-, -CC-, or -$Cx_{(2–3)}$C- motifs (middle); or in cytosolic regions of predicted protein disorder (bottom; determined using multiple algorithms on the DisProt server, http://www.disprot.org/metapredictor.php; Sickmeier et al., 2007) for transmembrane ion channel pore-forming subunits.

different zDHHCs function at different stages of the protein lifecycle, remains to be determined. Although systematic siRNA screens have, to date, not been performed on other ion channels, data from other multiply S-acylated channels, such as NMDA, AMPA, and BK channel subunits, supports the hypothesis that zDHHCs can show substrate specificity (Hayashi et al., 2005, 2009; Tian et al., 2010).

It is generally assumed that S-acylation facilitates the membrane association of protein domains. This is clearly the case for peripheral membrane proteins, such as RAS or PSD-95, but direct experimental evidence for S-acylation controlling membrane association of the cytosolic domains of transmembrane proteins is largely elusive. One of the best examples involves the large C-terminal domain of the BK channel, which comprises more than two-thirds of the pore-forming subunit (Fig. 5). In the absence of S-acylation of the STREX domain, or exclusion of the 59–amino acid STREX insert, the BK channel C terminus is cytosolic (Tian et al., 2008). However, if the STREX domain is S-acylated, the entire C terminus associates with the plasma membrane, a process that can be dynamically regulated by phosphorylation of a serine immediately upstream of the S-acylated cysteines in the STREX domain (Tian et al., 2008). This S-acylation–dependent membrane association markedly affects the properties and regulation of the channel (Jeffries et al., 2012) and has been proposed to confer significant structural rearrangements. In support of such structural rearrangement, S-acylated STREX channels are not inhibited by PKC-dependent phosphorylation even though a PKC phosphorylation site serine motif, conserved in other BK channel variants, is present downstream of the STREX domain. In other BK channel variants lacking the STREX insert, this PKC site is required for channel inhibition by PKC-dependent phosphorylation. However, after deacylation of the STREX domain, PKC can now phosphorylate this PKC phosphorylation serine motif, which suggests that the site has become accessible, consequently resulting in channel inhibition (Fig. 5; Zhou et al., 2012).

How might S-acylation of a cysteine residue juxtaposed to another membrane anchoring domain control protein function? The simplest mechanism would involve acting as an additional anchor (Fig. 3 A). In some systems, juxta-transmembrane palmitoylation allows tilting of transmembrane domains, effectively shortening the transmembrane domain to reduce hydrophobic mismatch (Nyholm et al., 2007), particularly at the thinner ER membrane (Abrami et al., 2008; Charollais and Van Der Goot, 2009; Baekkeskov and Kanaani, 2009), and confer conformational restraints on the peptide (Fig. 3 A). Such a mechanism has been proposed to control ER exit of the regulatory β4 subunits of BK channels. In this case, depalmitoylation of a cysteine residue juxtaposed to the second transmembrane domain of the β4 subunits may result in hydrophobic mismatch at the ER, reducing ER exit, and yield a conformation that is unfavorable for interaction with BK channel α subunits, thereby decreasing surface expression of BK channel α subunits (Chen et al., 2013).

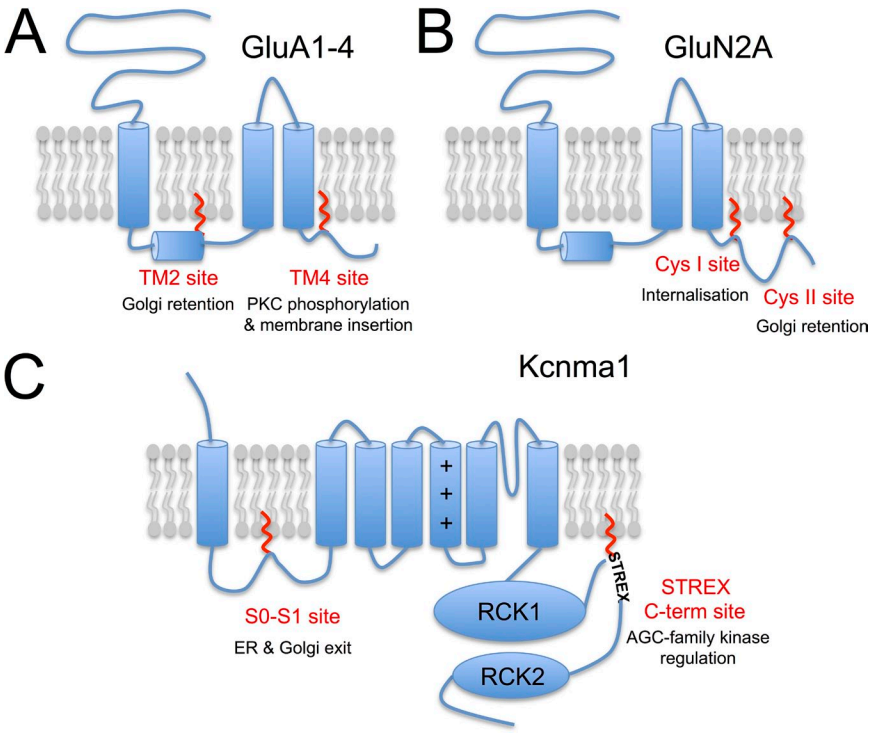

Figure 4. Multisite S-acylation in ion channels controls distinct functions. (A–C) Schematic illustrating location of multiple S-acylated domains in AMPA receptor GluA1–4 subunits (A), NMDA receptor GluN2A subunits (B), and BK channel pore-forming α subunits (C), encoded by the *Kcnma1* gene. Each domain confers distinct functions/properties on the respective ion channel and is regulated by distinct zDHHCs (see the "Control of ion channel cell surface expression and spatial organization in membranes" section for further details).

Tools to analyze ion channel S-acylation

Before the seminal discovery of the mammalian enzymes that control S-acylation (Fukata et al., 2004) and current advances in proteomic techniques to assay S-acylation, progress in the field was relatively slow, largely because of the lack of pharmacological, proteomic, and genetic tools to investigate the functional role of S-acylation. It is perhaps instructive to consider that protein tyrosine phosphorylation was discovered the same year as S-acylation (Hunter, 2009). However, the subsequent rapid identification and cloning of tyrosine kinases provided a very extensive toolkit to investigate this pathway. Although the S-acylation toolkit remains limited, the last few years have seen rapid progress in our ability to interrogate S-acylation function and its control of ion channel physiology. Furthermore, S-acylation prediction algorithms, such as CSS-palm 4.0 (Ren et al., 2008), provide an in silico platform to inform experimental approaches for candidate targets.

Pharmacological tools. The S-acylation pharmacological toolkit remains, unfortunately, empty, with limited specific agents with which to explore S-acylation function in vitro or in vivo. Although the palmitate analogue 2-bromopalmitate (2-BP) is widely used for cellular assays and to analyze ion channel regulation by S-acylation, caution must be taken in using this agent, even though it remains our best pharmacological inhibitor of zDHHCs (Resh, 2006; Davda et al., 2013; Zheng et al., 2013). Unfortunately, 2-BP is a nonselective inhibitor of lipid metabolism and many membrane-associated enzymes, and displays widespread promiscuity (e.g., Davda et al., 2013); does not show selectivity toward specific zDHHC proteins (Jennings et al., 2009); has many pleiotropic effects

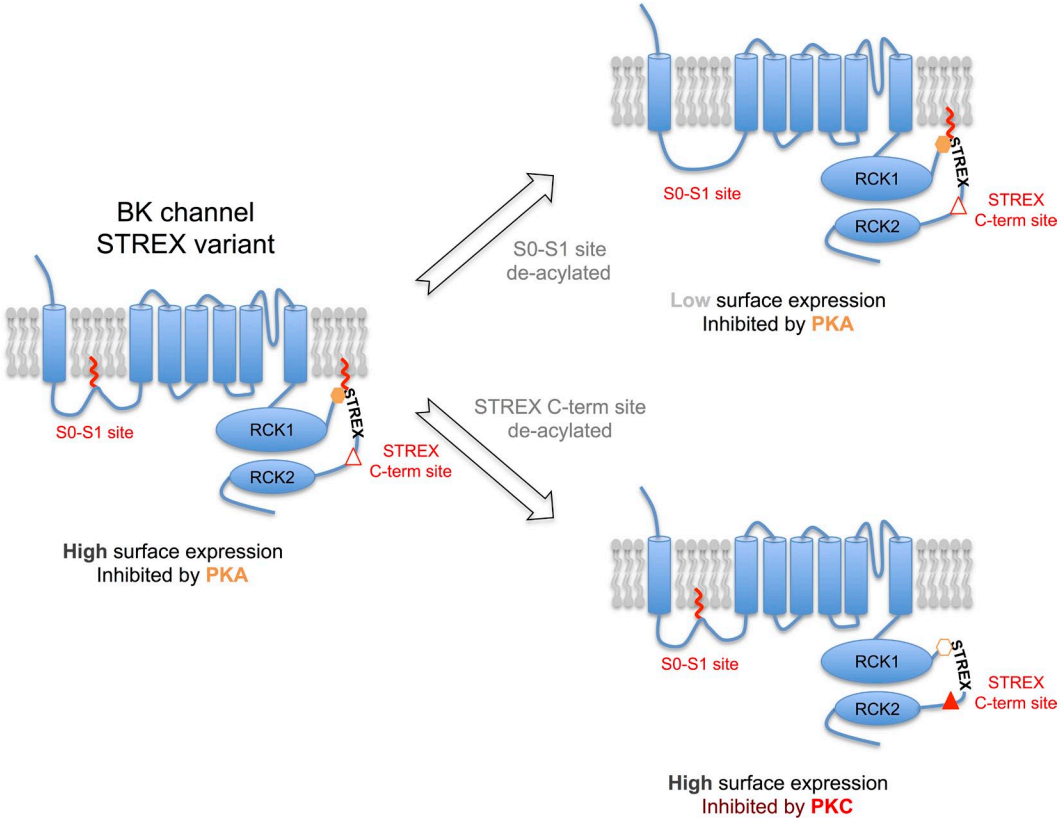

Figure 5. S-acylation controls BK channel trafficking and regulation by AGC family protein kinases via distinct sites. The BK channel STREX splice variant pore-forming α subunit is S-acylated at two sites: the S0-S1 loop and the STREX domain in the large intracellular C terminus. S-acylation of the S0-S1 loop promotes high surface membrane expression of the channel; thus, deacylation of this site decreases the number of channels at the cell surface (see the "Control of ion channel cell surface expression and spatial organization in membranes" section for further details). In contrast, S-acylation of the STREX domain allows inhibition of channel activity by PKA-mediated phosphorylation of a PKA serine motif (closed hexagon) immediately upstream of the palmitoylated cysteine residues in STREX. In the S-acylated state, PKC has no effect on channel activity even though a PKC phosphorylation site serine motif is located immediately downstream of the STREX domain (open triangle). Deacylation of STREX dissociates the STREX domain from the plasma membrane, and exposes the PKC serine motif so that it can now be phosphorylated by PKC (closed triangle), resulting in channel inhibition. In the deacylated state, PKA has no effect on channel activity (open hexagon). Thus, deacylation of the STREX domain switches channel regulation from a PKA-inhibited to a PKC-inhibited phenotype (see the "S-acylation and posttranslational cross-talk controls channel trafficking and activity" section for further details).

on cells at high concentrations, including cytotoxicity (Resh, 2006); and also inhibits acylthioesterases (Pedro et al., 2013). Other lipid inhibitors include cerulenin and tunicamycin. However, cerulenin affects many aspects of lipid metabolism, and tunicamycin inhibits N-linked glycosylation (Resh, 2006). Although some nonlipid inhibitors have been developed, these are not widely used (Ducker et al., 2006; Jennings et al., 2009), and there are currently no known activators of zDHHCs or compounds that inhibit specific zDHHCs. In the last few years, several inhibitors for the acylthioesterases LYPLA1 and LYPLA2 have been developed (Bachovchin et al., 2010; Dekker et al., 2010; Adibekian et al., 2012). However, several of these compounds, such as palmostatin B, are active against several members of the larger serine hydrolase family. Clearly, the development of novel S-acylation inhibitors and activators that display both specificity and zDHHC selectivity would represent a substantial advance for investigation of channel S-acylation.

Genetic tools. To date, most studies have used overexpression of candidate zDHHCs in heterologous expression or native systems and analyzed increases in [^{3}H]palmitate incorporation to define zDHHCs that may S-acylate specific ion channels (e.g. Rathenberg et al., 2004; Hayashi et al., 2005, 2009; Tian et al., 2010; Thomas et al., 2012). Although this is a powerful approach, caution is required to determine whether results obtained with overexpression in fact replicate endogenous regulation. For example, overexpression of some zDHHCs normally expressed in the cell type of interest can result in S-acylation of a cysteine residue that is not endogenously palmitoylated in BK channels (Tian et al., 2010). Point mutation of the cysteine of the catalytic DHHC domain abolishes the acyltransferase activity of zDHHCs and is thus an invaluable approach to confirming that the acyltransferase function of overexpressed zDHHC is required by itself. Increasingly, knockdown of endogenous zDHHCs using siRNA, and related approaches, is beginning to reveal the identity of zDHHCs that S-acylate native ion channel subunits. For example, knockdown of zDHHCs 5 or 8 reduces S-acylation of the accessory subunits PICK1 and Grip1, which control AMPA receptor trafficking (Thomas et al., 2012, 2013); and knockdown of zDHHC2 disrupts local nanoclusters of the PDZ domain protein PSD-95 in neuronal dendrites to control AMPA receptor membrane localization (Fukata et al., 2013). However, relatively few studies have taken a systematic knockdown approach to identify zDHHCs important for ion channel S-acylation. One such approach has, however, revealed that multiple, distinct zDHHCs mediate palmitoylation of the BK channel C terminus (zDHHCs 3, 5, 7, 9, and 17) and that a different subset of zDHHCs (22 and 23) mediate S-acylation of the intracellular S0-S1 loop in the same channel (Tian et al., 2010, 2012). Because some zDHHCs are themselves palmitoylated, the functional effect of overexpressing or knocking down individual zDHHCs on the localization and activity of other zDHHCs must also be carefully determined. For example, siRNA-mediated knockdown of zDHHC 5, 7, or 17 in HEK293 cells paradoxically results in an up-regulation of zDHHC23 mRNA expression (Tian et al., 2012). Furthermore, because many signaling and cytoskeletal elements are also controlled by S-acylation, direct effects on channel S-acylation by themselves must be evaluated in parallel (for example using site-directed cysteine mutants of the channel subunit). Fewer studies have used these approaches to examine the role of acylthioesterases, although overexpression of LYPLA1 and a splice variant of LYPLAL1, but not LYPLA2, deacylates the S0-S1 loop of the BK channel, promoting Golgi retention of the channels (Tian et al., 2012). Gene-trap and knockout mouse models for some zDHHCs (such as 5 and 17) are becoming available, although full phenotypic analysis and analysis of ion channel function in these models are largely lacking.

Proteomic and imaging tools. Lipid-centric (metabolic) labeling assays. Metabolic labeling approaches are most suited to analysis of isolated cells, rather than tissues, but provide information on dynamic palmitoylation of proteins during the relatively short (~4 h) labeling period as well as insight into the species of lipid bound to cysteine residues. The classical approach using radioactive palmitate (e.g., [^{3}H]palmitate) remains a "gold standard" for validation, in particular for identification that palmitate is the bound lipid. However, metabolic labeling with [^{3}H]palmitate generally requires immunoprecipitation and days to weeks of autoradiography or fluorography, particularly when analyzing low abundance membrane proteins such as ion channels. To overcome some of these issues, and also to provide a platform to allow cellular imaging of S-acylation, a variety of biorthogonal lipid probes have recently been developed (Hannoush and Arenas-Ramirez, 2009; Hannoush, 2012; Martin et al., 2012; for reviews see Charron et al., 2009a; Hannoush and Sun, 2010). These probes are modified fatty acids with reactive groups, such as an azide or alkyne group, allowing labeled proteins to be conjugated to biotin or fluorophores via the reactive group using Staudinger ligation or "click" chemistry. In particular, development of a family of ω-alkynyl fatty acid probes of different chain lengths (such as Alk-C16 and Alk-C18) have been exploited for proteomic profiling as well as single cell imaging (Gao and Hannoush, 2014) and have been used to identify candidate S-acylated channels in several mammalian cell lines (Table 3; Charron et al., 2009b; Hannoush and Arenas-Ramirez, 2009; Martin and Cravatt, 2009; Yap et al., 2010; Yount et al., 2010; Martin et al., 2012). It is important to note that palmitic acid can also be incorporated into free N-terminal cysteines of proteins via an amide linkage (N-palmitoylation), addition of the monounsaturated palmitoleic acid via an oxyester linkage to a serine residue (O-palmitoylation),

and oleic acid (oleoylation) as well as myristate via amide linkages on lysine residues (Stevenson et al., 1992; Linder and Deschenes, 2007; Hannoush and Sun, 2010; Schey et al., 2010). These modifications can be discriminated from S-acylation by their insensitivity to hydroxylamine cleavage (at neutral pH) compared with the S-acylation thioester linkage. Whether N- or O-linked palmitoylation or oleoylation controls ion channel function remains to be determined.

Cysteine centric (cysteine accessibility) assays: Acyl-biotin exchange (ABE) and resin-assisted capture (Acyl-RAC). The metabolic labeling approach requires treating isolated cells with lipid conjugates and thus largely precludes analysis of native S-acylation in tissues. However, several related approaches have been developed that exploit the exposure of a reactive cysteine after hydroxylamine cleavage (at neutral pH) of the cysteine-acyl thioester linkage. The newly exposed cysteine thiol can then react with cysteine-reactive groups (such as biotin-BMCC or biotin-HPDP used in the ABE approach; Drisdel and Green, 2004; Drisdel et al., 2006; Draper and Smith, 2009; Wan et al., 2007) or thiopropyl sepharose (used in Acyl-RAC; Forrester et al., 2011) to allow purification of S-acylated proteins that can be identified by Western blot analysis or mass spectrometry. Acyl-RAC has been reported to improve detection of higher molecular weight S-acylated proteins and thus may prove valuable for ion channel analysis. These approaches have been exploited to determine the "palmitoylome" in several species and tissues (e.g., Table 3; Wan et al., 2007, 2013; Kang et al., 2008; Martin and Cravatt, 2009; Yang et al., 2010; Yount et al., 2010; Merrick et al., 2011; Wilson et al., 2011; Jones et al., 2012; Ren et al., 2013). For example, analysis of rat brain homogenates identified both previously characterized as well as novel S-acylated ion channels (Tables 1–3). ABE and Acyl-RAC have recently been adapted to allow more quantitative labeling in vivo for comparative assays (Wan et al., 2013), although it must be remembered that these approaches detect S-acylation and do not define S-palmitoylation per se. Cysteine accessibility approaches determine the net amount of preexisting S-acylated proteins; however, caution is required to eliminate false positives. In particular it is necessary to fully block all reactive cysteines before hydroxylamine cleavage; moreover, the identity of the endogenously bound lipid is of course not known.

The lipid- and cysteine-centric approaches are thus complementary. In conjunction with site-directed mutagenesis of candidate S-acylated cysteine residues in ion channel subunits, these approaches have provided substantial insight into the role and regulation of ion channel S-acylation (Tables 1–3). However, tools that are widely accessible for examination of other post-translational modifications of ion channels (such as phosphospecific antibodies to analyze phosphoproteins in cells and tissues) are largely lacking in the S-acylation field. As a result, for example, spatial analysis of native S-acylation in cells or tissue sections is largely lacking. Recently, an intrabody that recognizes the conformational rearrangement of the ion channel scaffolding protein PSD-95 upon S-acylation, allowing cycles of S-acylation and deacylation of native PSD-95 to be monitored in neurons, has recently been developed (Fukata et al., 2013). However, this approach does not directly confirm that the protein is S-acylated per se. Furthermore, in most ion channels, and in fact most S-acylated proteins, the identity of the native lipid bound to a specific S-acylated cysteine is not known. Although palmitate is considered to be the major lipid species involved in S-acylation, this has not been directly demonstrated in most cases, and other fatty acids, including arachidonic acid, oleate acid, and stearic acid, have also been reported to bind to cysteine via a thioester S-linkage (Linder and Deschenes, 2007; Hannoush and Sun, 2010). A major reason for this discrepancy is that mass spectrometry–based approaches to identify the native lipid specifically bound to S-acylated cysteines remain a significant challenge. This is particularly true for low abundance proteins such as mammalian ion channels, in contrast to the widespread application of mass spectrometry to directly identify native amino acids that are phosphorylated (Kordyukova et al., 2008, 2010; Sorek and Yalovsky, 2010; McClure et al., 2012; Ji et al., 2013). As such, direct biochemical demonstration of native cysteine S-acylation is lacking in most ion channels.

S-acylation and control of the ion channel lifecycle

Ion channel physiology is determined by both the number of channel proteins at the cognate membrane and by their activity and/or kinetics at the membrane. Evidence has begun to emerge that S-acylation of either pore-forming or regulatory subunits of ion channels controls all of these aspects of ion channel function. Although the focus of this review is S-acylation–dependent regulation of ion channel subunits itself, S-acylation also regulates the localization or activity of many adaptor, scaffolding, and cellular signaling proteins (e.g., G protein–coupled receptors [GPCRs], AKAP18, AKAP79/150, G proteins, etc.), as well as other aspects of cell biology that affect ion channel trafficking and the activity and regulation of macromolecular ion channel complexes (El-Husseini and Bredt, 2002; Linder and Deschenes, 2007; Fukata and Fukata, 2010; Greaves and Chamberlain, 2011; Shipston, 2011; Resh, 2012).

Control of ion channel cell surface expression and spatial organization in membranes. The control of ion channel trafficking, from synthesis in the ER through modification in the Golgi apparatus to subsequent delivery to the appropriate cellular membrane compartment, is a

major mechanism whereby S-acylation modulates ion channel physiology. S-acylation may influence the number of ion channels resident in a membrane through regulation of distinct steps in the ion channel lifecycle (Fig. 2). Indeed S-acylation has been implicated in ion channel synthesis, as well as in channel trafficking to the membrane and subsequent internalization, recycling, and degradation. S-acylation controls the maturation and correct assembly of ion channels early in the biosynthetic pathway. For example, S-acylation regulates assembly of the ligand gated nicotinic acetylcholine receptor (nAChR) to ensure a functional binding site for acetylcholine (Alexander et al., 2010) as well as controlling its surface expression (Amici et al., 2012). S-acylation is also an important determinant of the maturation of both voltage-gated sodium (Nav1.2) and voltage-gated potassium channels (Kv1.5; Schmidt and Catterall, 1987; Zhang et al., 2007). S-acylation also contributes to the efficient trafficking of channels from the ER to Golgi and to post-Golgi transport. Three examples illustrate the importance and potential complexity of S-acylation in controlling ion channel trafficking:

(1) S-acylation of a cysteine residue adjacent to a hydrophobic region (TM2) in a cytosolic loop of the GluA1 pore-forming subunit of AMPA receptors (Fig. 4 A) promotes retention of the channel in the Golgi (Hayashi et al., 2005). However, S-acylated Grip1b, a PDZ protein that binds to AMPA receptors, is targeted to mobile trafficking vesicles in neuronal dendrites and accelerates local recycling of AMPA receptors to the plasma membrane (Thomas et al., 2012). In contrast, S-acylation of another AMPA receptor interacting protein, PICK1, is proposed to stabilize AMPA receptor internalization (Thomas et al., 2013).

(2) S-acylation of a cluster of cysteine residues juxtaposed to the transmembrane 4 domain (Cys I site) of the NMDA receptor subunit GluN2A (Fig. 4 B) increases surface expression of NMDA receptors by decreasing their constitutive internalization. In contrast S-acylation at C-terminal cysteine residues (Cys II site) decreases their surface expression by introducing a Golgi retention signal that decreases forward trafficking (Hayashi et al., 2009). Even though both sites affect surface expression, only S-acylation of the TM4 juxtaposed cysteine residues influences synaptic incorporation of NMDA receptors, which suggests that this site is an important determinant of the synaptic versus extrasynaptic localization of these ion channels (Mattison et al., 2012). Together, these data highlight the importance of S-acylation of two distinct sites within the same ion channel as well as that of components of the ion channel multimolecular complex as determinants of channel trafficking.

(3) S-acylation of a cluster of cysteine residues in the intracellular S0-S1 loop of the pore-forming subunit (Figs. 4 C and 5) is required for efficient exit of BK channels from the ER and the trans-Golgi network. Deacylation at the Golgi apparatus appears to be an important regulatory step (Tian et al., 2012). BK channel surface abundance may also be controlled by S-acylation of regulatory β4 subunits. β4 subunit S-acylation on a cysteine residue juxtaposed to the second transmembrane domain is important for the ability of the β4 subunit itself to exit the ER. Importantly, assembly of β4 subunits with specific splice variants of pore-forming α subunits of the BK channel enhances surface expression of the channel, a mechanism that depends on S-acylation of the β4 subunit (Chen et al., 2013). Thus, in BK channels, S-acylation of the S0-S1 loop of the pore-forming subunit controls global BK channel surface expression, and β4 subunit S-acylation controls surface expression of specific pore-forming subunit splice variants. S-acylation of the Kchip 2 and Kchip 3 accessory subunits also controls surface expression of voltage-gated Kv4.3 channels (Takimoto et al., 2002).

Moreover, S-acylation modulates the spatial organization of ion channels within membranes. Perhaps the most striking example involves aquaporin 4 (AQP4), where S-acylation of two N-terminal cysteine residues in an N-terminal splice variant (AQP4M1) inhibits assembly of AQP4 into large orthogonal arrays (Suzuki et al., 2008; Crane and Verkman, 2009), perhaps by disrupting interactions within the AQP4 tetramer. S-acylation can affect the distribution of the many membrane-associated proteins between cholesterol-rich microdomains (lipid rafts) and the rest of the membrane. Such clustering has also been reported for various transmembrane proteins, including the P2x purinoceptor 7 (P2X7) receptor, in which S-acylation of the C terminus promotes clustering into lipid rafts (Gonnord et al., 2009). A similar mechanism may underlie synaptic clustering of $GABA_A$ receptors mediated by S-acylation of an intracellular loop of the y2 subunit (Rathenberg et al., 2004). In these examples, S-acylation of the channel itself affects membrane partitioning and organization. However, recent evidence in neurons suggests that establishment of "nano" domains of ion channel complexes in postsynaptic membranes may also be established by local clustering of the cognate acyltransferase itself. For example, clustering of zDHHC2 in the postsynaptic membranes of individual dendritic spines provides a mechanism for local control of S-acylation cycles of the PDZ protein adapter, PSD-95, and thereby for controlling its association with the plasma membrane. PSD-95, in turn, can assemble with various ion channels, including NMDA receptors, and can thus dynamically regulate the localization and clustering of ion channel complexes (Fukata et al., 2013). Indeed, an increasing number of other ion channel scaffolding proteins such as Grip1 (Thomas et al., 2012), PICK1 (Thomas et al., 2013), S-delphilin (Matsuda et al., 2006), and Ankyrin G (He et al., 2012) that influence ion channel trafficking, clustering, and localization are now known to be S-acylated.

Relatively few studies have identified effects of S-acylation on the intrinsic gating kinetics or pharmacology of ion channels at the plasma membrane. However, a glycine-to-cysteine mutant (G1079C) in the intracellular loop between domains II and III enhances the sensitivity of the voltage-gated Na channel Nav1.2a to the toxins PaurTx3 and ProTx-II, an effect blocked by inhibition of S-acylation. These toxins control channel activation through the voltage sensor in domain III. In addition, deacylation of another (wild-type) cysteine residue (C1182) in the II–III loop produces a hyperpolarizing shift in both activation and steady-state inactivation as well as slowing the recovery from fast inactivation and increasing sensitivity to PaurTx3 (Bosmans et al., 2011). Effects of S-acylation on gating kinetics have also been reported in other channels. For example, in the voltage-sensitive potassium channel Kv1.1, S-acylation of the intracellular linker between transmembrane domains 2 and 3 increases the intrinsic voltage sensitivity of the channel (Gubitosi-Klug et al., 2005). S-acylation of the β and γ subunits of epithelial sodium channels (ENaC) also affects channel gating (Mueller et al., 2010; Mukherjee et al., 2014), and the S-acylated regulatory β2a subunit of N-type calcium channels controls voltage-dependent inactivation (Qin et al., 1998; Hurley et al., 2000).

S-acylation is also an important determinant of retrieving ion channels from the plasma membrane for recycling or degradation. S-acylation of a single cysteine residue juxtaposed to the transmembrane TM4 domain of GluA1 and GluA2 subunits of AMPA receptors controls agonist-induced ion channel internalization. These residues are distinct from those controlling Golgi retention of AMPA receptors (Fig. 4 A), which emphasizes the finding that the location and context of the S-acylated cysteines, even in the same protein, is central for their effects on physiological function (Hayashi et al., 2005; Lin et al., 2009; Yang et al., 2009). The stability of many proteins is also regulated by S-acylation; S-acylation of a single cysteine residue in Kv1.5 promotes both its internalization and its degradation (Zhang et al., 2007; Jindal et al., 2008). Thus, in different ion channels, S-acylation can have opposite effects on insertion, membrane stability, and retrieval.

S-acylation and posttranslational cross-talk control channel trafficking and activity. An emerging concept is that S-acylation is an important determinant of ion channel regulation by other PTMs. Indeed, nearly 20 years ago it was reported that PKC-dependent phosphorylation of the GluK2 (GluR6) subunit of Kainate receptors was attenuated in channels S-acylated at cysteine residues near the PKC consensus site (Pickering et al., 1995). S-acylation of GluA1 subunits of AMPA receptors also blocks PKC phosphorylation of GluA1 and subsequently prevents its binding to the cytoskeletal adapter protein 4.1N, ultimately disrupting AMPA receptor insertion into the plasma membrane (Lin et al., 2009). Intriguingly, PKC phosphorylation and S-acylation have the opposite effect on 4.1N-mediated regulation of Kainate receptor (GluK2 subunit) membrane insertion: in this, case S-acylation promotes 4.1N interaction with Kainate receptors and thereby receptor insertion, whereas PKC phosphorylation disrupts 4.1N interaction, promoting receptor internalization (Copits and Swanson, 2013). Disruption of phosphorylation by S-acylation of residues near consensus phosphorylation sites likely results from steric hindrance, as proposed for S-acylation–dependent regulation of β2 adrenergic receptor phosphorylation (Mouillac et al., 1992; Moffett et al., 1993).

S-acylation has also been reported to promote ion channel phosphorylation. For example, site-directed mutation of a cluster of palmitoylated cysteine residues in the GluN2A subunit of NMDA receptors abrogates Fyn-dependent tyrosine phosphorylation at a site between TM4 and the palmitoylated cysteines (Hayashi et al., 2009). Therefore, S-acylation of GluN2A promotes tyrosine phosphorylation, resulting in reduced internalization of the NMDA receptor (Hayashi et al., 2009). Furthermore, S-acylation of BK channels can act as a gate to switch channel regulation to different AGC family kinase signaling pathways, emphasizing the complex interactions that can occur between signaling pathways (Tian et al., 2008; Zhou et al., 2012; Fig. 5). S-acylation of an alternatively spliced insert (STREX) in the large cytosolic domain of the pore-forming subunit of BK channels promotes association of the STREX domain with the plasma membrane. S-acylation of the STREX insert is essential for the functional inhibition of STREX BK channels by PKA-mediated phosphorylation of a serine residue immediately upstream of the S-acylated cysteines. PKA phosphorylation dissociates the STREX domain from the plasma membrane (Tian et al., 2008), preventing STREX domain S-acylation (Jeffries et al., 2012) and leading to channel inhibition. However, deacylation of the STREX domain exposes a PKC consensus phosphorylation site downstream of the STREX domain, allowing PKC to inhibit STREX BK channels (Zhou et al., 2012). Thus, S-acylation acts as a reversible switch to specify regulation by AGC family kinases through control of the membrane association of a cytosolic domain of the channel: S-acylated STREX BK channels are inhibited by PKA but insensitive to PKC, whereas deacylated channels are inhibited by PKC but not PKA (Fig. 5). The reciprocal control of membrane association of a protein domain by S-acylation and protein phosphorylation likely represents a common mechanism in other signaling proteins as revealed for phosphodiesterase 10A (Charych et al., 2010).

Cysteine residues are targets for several other modifications that regulate various ion channels, including nitrosylation, sulphydration, REDOX regulation, and formation of disulphide bonds (Sen and Snyder, 2010).

Evidence is beginning to emerge that S-acylation may mutually compete with these mechanisms, providing a dynamic network to control cysteine reactivity. For example, the ion channel scaffolding PDZ domain protein PSD-95 is S-acylated at two N-terminal cysteine residues (C3 and C5) that are required for membrane targeting and clustering of PSD-95 (El-Husseini et al., 2002). nNOS also interacts with PSD-95, and stimulation of nitric oxide production results in nitrosylation of these cysteines, preventing their S-acylation and thereby decreasing PSD-95 clusters at postsynaptic sites (Ho et al., 2011). A recent remarkable example of the potential for such cross-talk in ion channel subunits is the identification of the S-acylation of 18 different cysteine residues in the large cytosolic N terminus of RyR1 in skeletal muscle. Of these 18 S-acylated cysteines, six have previously been identified as targets for S-oxidation, and a further cysteine residue was also subject to S-nitrosylation (Chaube et al., 2014) Although the functional relevance of this potential cross-talk in RyR1 has yet to be defined, interaction between oxidation and S-acylation of the same cysteine residue is physiologically relevant in other proteins. For example, oxidation of the signaling protein HRas at two cysteine residues C181/184 prevents S-acylation of these residues, resulting in a loss of plasma membrane localization of this peripheral membrane signaling protein (Burgoyne et al., 2012). Intriguingly, a conserved cysteine residue in nAChR α3 subunits, which has been shown to be S-acylated (C273) in the nAChR α4 subunit, has been implicated in use-dependent inactivation of nAChRs by reactive oxygen species (Amici et al., 2012). Determining whether these mutually competitive cysteine modifications represent an important mechanism for regulation of a range of ion channels is an exciting challenge for the future.

S-acylation is also an important determinant of ion channel regulation by heterotrimeric G proteins. This can involve S-acylation of either G protein targets or of regulators of G proteins. In an example of the former, the palmitoylated N terminus of the regulatory β2a subunit splice variant acts as a steric inhibitor of an arachidonic acid binding domain to stimulate N-type calcium channels (Chien et al., 1996; Heneghan et al., 2009; Mitra-Ganguli et al., 2009). When the regulatory β subunits are not S-acylated, however, Gq-mediated signaling, via arachidonic acid, inhibits calcium channel activity. Closure of G protein regulated inward rectifying potassium (GIRK) channels in neurons after Gi/o deactivation provides an example of the latter (Jia et al., 2014). Signaling by members of the Gi/o family of the Gα subunit of heterotrimeric G proteins is terminated by members of the regulator of G protein signaling 7 (R7 RGS) family of GTPase-activating proteins, which accelerate GTP hydrolysis to speed Gi/o deactivation. Membrane localization of regulator of G protein signaling 7 (R7-RGS) is required for its regulation of Gi/o, and this is determined by interaction with an S-acylated R7 binding protein (R7-BP) that acts as an allosteric activator. Thus, the R7-RGS complex, recruited to the plasma membrane by S-acylated R7-BP, promotes Gi/o deactivation to facilitate GIRK channel closure. Conversely, deacylation of R7-BP removes the R7-GS complex from the plasma membrane, slowing Gi/o deactivation and consequent channel closure (Jia et al., 2014). Clearly, as S-acylation can also control an array of GPCRs, enzymes, and signaling and adapter proteins that indirectly control ion channel function (El-Husseini and Bredt, 2002; Linder and Deschenes, 2007; Fukata and Fukata, 2010; Greaves and Chamberlain, 2011; Shipston, 2011; Resh, 2012), understanding how S-acylation dynamically controls other components of ion channel multimolecular signaling complexes will be an essential future goal.

Summary and perspectives

With an ever-expanding "catalog" of S-acylated ion channel pore-forming and regulatory subunits (∼50 to date), together with an array of S-acylated scaffolding and signaling proteins, the importance and ubiquity of this reversible covalent lipid modification in controlling the lifecycle and physiological function and regulation of ion channels is unquestionable. This has been paralleled by a major resurgence in the wider S-acylation field, a consequence in large part of the discovery of S-acylating and deacylating enzymes together with a growing arsenal of genetic, proteomic, imaging, and pharmacological tools to assay and interrogate S-acylation function.

As for most other posttranslational modifications of ion channels, including phosphorylation, major future goals for the field include:

(1) Understanding mechanistically how covalent addition of a fatty acid can control such a diverse array of ion channel protein properties and functions, and how this is spatiotemporally regulated.

(2) Elucidating the physiological relevance of this posttranslational modification from the level of single ion channels to the functional role of the channel in the whole organism in health and disease.

Elucidation of these issues has fundamental implications far beyond ion channel physiology.

To address these goals several major challenges and questions must be addressed, including:

(1) It is largely assumed that S-acylation of transmembrane proteins results in an additional "membrane anchor" to target domains to the membrane interface. However, understanding the mechanisms, forces, and impact of S-acylation on the orientation of transmembrane helices and the architecture and structure of disordered domains in cytosolic loops and linkers, while remaining a considerable technical challenge, should provide major insight into mechanisms controlling channel trafficking, activity, and regulation.

(2) Although S-acylation is widely accepted to be reversible, its spatiotemporal regulation of most ion channels is unknown. Mechanistic insight into zDHHC and acylthioesterase substrate specificity, native subcellular localization, and assembly with ion channel signaling complexes will allow us to dissect and understand how S-acylation of ion channels is controlled. Importantly, this should allow us to take both "channel-centric" (e.g., site-directed mutagenesis of S-acylated cysteines) as well as "S-acylation centric" (e.g., knockout of specific zDHHC activity) approaches to understand how multisite S-acylation on the same ion channel subunit can control distinct functions as well as physiological regulation of trafficking and function at the plasma membrane.

(3) The functional role of S-acylation cannot be viewed in isolation from other posttranslational modifications. The cross-talk between S-acylation and adjacent phosphorylation sites as well as other cysteine modifications highlights the importance of understanding the interactions between signaling pathways. Insight into the rules, mechanisms, and cross-talk of S-acylation with these modifications has broad implications for cellular signaling.

(4) Although it is clear that disruption of S-acylation homeostasis itself has substantial effects on normal physiology, and we are beginning to understand some of the cellular functions of ion channel S-acylation, we know very little about the functional impact of disrupted ion channel S-acylation at the systems and organismal level. Understanding how this may be dynamically regulated during a lifespan is critical to understanding the role of S-acylation in health and disease.

To address these issues, development of improved tools to assay and investigate S-acylation from the single protein to organism is required. For example, tools to allow the real-time analysis of S-acylation status of ion channels in cells and tissues will provide fundamental insights into its dynamics and role in ion channel trafficking and membrane localization. Improved proteomic tools will allow direct assay of fatty acids bound to cysteine residues via thioester linkages. Development of new tools and models are essential if we are to understand the physiological relevance of ionic channel S-acylation at the systems level. These include: specific inhibitors of zDHHCs and thioesterases, conditional knockouts to spatiotemporally control zDHHC expression, and transgenics expressing catalytically inactive zDHHCs and models expressing S-acylation–null ion channel subunits. Furthermore, our understanding of how S-acylation may be dynamically controlled during normal ageing in response to homeostatic challenge and disruption in disease states remains rudimentary. Whether we will start to uncover channel "S-acylationopathies" resulting from dysregulation of ion channel S-acylation, analogous to channel phosphorylopathies, remains to be explored. Addressing these issues, together with development of new tools, will provide a paradigm shift in our understanding of both ion channel and S-acylation physiology, and promises to reveal novel therapeutic strategies for a diverse array of disorders.

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