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Trafficking of intermediate (KCa3.1) and small (KCa2.x) conductance, Ca²⁺-activated K⁺ channels: A novel target for medicinal chemistry efforts?

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Abstract

Ca²⁺-activated K⁺ channels (KCa) play a pivotal role in the physiology of a wide variety of tissues and disease states, including vascular endothelia, secretory epithelia, certain cancers, red blood cells (RBC), neurons and immune cells. Such widespread involvement has generated an intense interest in elucidating the function and regulation of these channels, with the goal of developing pharmacological strategies aimed at selective modulation of KCa channels in various disease states. Herein, we give an overview of the molecular and functional properties of these channels and their therapeutic importance as well as discuss the achievements made in designing pharmacological tools which control the function of KCa channels by modulating their gating properties. Moreover, this review discusses the recent advances in our understanding of KCa channel assembly and anterograde trafficking toward the plasma membrane, the microdomains in which these channels are expressed within the cell and finally the retrograde trafficking routes these channels take following endocytosis. As both the regulation of intracellular trafficking by agonists, as well as the protein-protein interactions that modify these events continue to be explored, we anticipate this will open up new therapeutic avenues for the targeting of these channels based on the pharmacological modulation of KCa channel density at the plasma membrane.

Keywords

KCa3.1; KCa2.1; KCa2.2; KCa2.3; channel density; trafficking; pharmacological modulators

Introduction

Homeostasis of mammalian cells is crucially dependent upon Ca²⁺ signaling pathways that result in proper cellular function. The intermediate- (KCa3.1, also called IK1 or SK4) and small (KCa2.1 (SK1), KCa2.2 (SK2), KCa2.3 (SK3))-conductance Ca²⁺-activated K⁺ channels of the KCNN gene family are widely distributed throughout the body, including brain, smooth muscle, epithelia and endothelia. Further, these channels are an important link between the modulation of membrane potential and the cellular Ca²⁺ signaling cascade, and thus, modulation of these channels influence the regulation of cellular function. As such, members of this gene family have been shown to play a crucial role in a host of physiological processes. Furthermore, through both the use of pharmacological modulators

as well as knockout and transgenic animal studies these channels have been implicated in a host of disease processes.

In this minireview, we provide a concise overview of the physiology of these channels and in particular focus on their potential role in the diseases process. We then address the medicinal chemistry efforts which have led to a relative wealth of pharmacological openers (activators) and blockers (inhibitors) for these channels. Indeed, just within the past 2–3 years exciting advances have occurred within this field such that high affinity and channel subtype specific compounds have been developed that decrease current flow by altering apparent Ca^{2+} -sensitivity rather than classically blocking ion flow. Of course, the flow of ions across a membrane is not only dependent upon the open probability (P_o), or gating of the channel, but also the number of channels (N) of any given type present at the membrane. Understanding how N may be dynamically modulated requires knowledge of both the anterograde and retrograde trafficking of these channels. While the regulation of channel gating by Ca^{2+} , kinases, fatty acids and pharmacological tools have been explored in detail, studies on the trafficking of KCa channels has been slower to gain a foothold. However, numerous recent reports have resulted in an acceleration of this field of study. Herein, we will examine these recent studies which have begun to define both the molecular motifs and accessory proteins required for the trafficking of KCa channels to the plasma membrane as well as their endocytic retrieval from the cell surface. Finally, we explore the possibility that pharmacological modulators, by altering the gating of KCa channels, may also alter plasma membrane residence time while also speculating on whether medicinal chemistry efforts designed to explicitly target the trafficking of KCa channels will prove therapeutically useful.

Small-conductance KCa2.x Channels

KCa2.x channels have been described in the literature for many years. Indeed, over 70 years ago, scientists reported a toxic action of bee venom, which we now know is apamin, when administered to the central nervous system [1]. Adelman and colleagues [2] originally cloned the small-conductance Ca^{2+} -activated K^+ channels (KCa2.1, KCa2.2 and KCa2.3, see Table 1) from rat and human brain. As schematically shown in Fig. 1, the KCa2.x channels exhibit a classic 6-transmembrane domain (S1–S6) architecture, with the re-entrant pore loop between S5 and S6 as well as cytoplasmic N- and C- termini [2]. There is considerable homology within the core of these channels (S1–S6); being approximately 80–90% [2]. This homology is extended to the proximal C-termini, just distal to S6, and it was quickly revealed that this region of the channel acted as a constitutive calmodulin binding domain (CMBD) [5]. It is the constitutive binding of calmodulin to the CMBD that imbues these channels with their submicromolar affinity for Ca^{2+} [5a, 6]. In contrast to these core regions, the homology of the remainder of the C-terminus and most of the N-terminus among the KCa2.x channels is much less [1b, 2, 5a]. The differences in homology between the N- and C-termini suggest that these portions of the KCa2.x channels convey physiological and functional differences apart from the Ca^{2+} sensitivity [1b]. These channels generally exist as homotetrameric channels [2], however, heterotetrameric channels have been reported in both native tissue [7] and heterologously expressed channels [8]. The electrophysiological examination of KCa2.x channels has been substantial; suffice to say, these channels have a relatively small conductance (5–15 pS), are voltage-independent, and exhibit an inwardly-rectified current-voltage relationship. The gating of these channels has been recently reviewed by Adelman and colleagues [1b] and will not be further discussed here.

Regulation of KCa2.x channels

As noted above, Adelman and colleagues recognized early on that the Ca^{2+} -dependent gating of KCa2.x channels was induced by the constitutive binding of one calmodulin to

each of the four subunits of the tetrameric channel such that the binding of Ca^{2+} to the N-lobe E-F hands of calmodulin induced a conformational change in the C-terminus of the channel; resulting in channel gating [5, 6c]. This was elegantly confirmed upon solving the crystal structure of the CMBD with calmodulin as it was revealed calcified calmodulin associates with two CMBD such that a dimeric organization is realized, whereas apo-calmodulin associates with a single CMBD [6c, 9]. A critical breakthrough in the understanding of KCa2.x regulation is that these channels exist as protein complexes such that kinases and phosphatases are associated with the channel (Fig. 1). In this regard, it has been demonstrated that KCa2.2 associates with both protein kinase CK2 as well as protein phosphatase 2A (PP2A) such that they phosphorylate and dephosphorylate the associated calmodulin, respectively resulting in a shift in the apparent Ca^{2+} -sensitivity of the channel [10]. Additionally, KCa2.x channels have been shown to be localized in close proximity to their source of activating Ca^{2+} , including voltage-gated Ca^{2+} channels (Cav), N-methyl d-aspartate (NMDA) receptors and nicotinic acetylcholine (nACh) receptors. In this way, voltage-dependent activation of Ca^{2+} entry through Cav or direct agonist-mediated Ca^{2+} entry via NMDA and nACh receptors triggers the activation of KCa2.x channels resulting in hyperpolarization. For additional information on this topic, we would direct the reader to an extensive recent review by Adelman and colleagues [1b].

Role of KCa2.x channels in normal physiology and disease

As mentioned, KCa2.x channels are widely distributed throughout the brain, muscle and endothelial cells, so it is not surprising that KCa2.x channels have a number of physiological functions. One of the primary functions of KCa2.x channels in neurons is contributing to the medium afterhyperpolarization (mAHP) that follows an action potential [1b, 11]. The action of the KCa2.x channels during the mAHP aids in preventing the initiation of a second action potential, thus, KCa2.x channels function as regulators of the frequency of action potential firing [1b]. Given this central role in controlling action potential firing frequency it is perhaps not surprising that KCa2.3 has been implicated in a number of neurological conditions including schizophrenia [12], anorexia nervosa [13] and ataxia [14]. Indeed, Chandy and colleagues identified a truncated KCa2.3 splice variant that acted as a dominant negative for other KCa2.x channels [12b] and when this was expressed exclusively in the deep cerebellar nuclei of mice it resulted in increased spontaneous action potential firing and ataxia [14b]. Further, activators of KCa2.2 have been shown to improve motor coordination in ataxic mice suggesting that KCa2.2 plays a crucial role in motor function [15]. This proposed role for KCa2.x channels in schizophrenia, ataxia and anorexia nervosa is a result of their function in controlling the activity of dopaminergic and serotonergic neurons [11]. Indeed, activation of KCa2.x channels by 1-EBIO¹ increased the AHP and decreased the firing frequency of dopaminergic neurons in midbrain slices [16] while suppressing bursting behavior *in vivo* [17], whereas inhibition of KCa2.3 channels shifts a tonic firing pattern to a burst pattern [11, 16]. Such shifts in firing pattern of dopaminergic neurons would result in altered release of dopamine with effects on motor control, working memory and reward-directed behaviour [11, 18]. Thus, modulators of KCa2.3 have also been proposed as

¹Abbreviations:

NS4591: (4,5-dichloro-1,3-diethyl-1,3-dihydro-benzimidazol-2-one); NS004: 5-trifluoromethyl-1-(5-chloro-2-hydroxyphenyl)-1,3-dihydro-2H-benzimidazol-2-one; NS1619: 1-(2'-hydroxy-5'-trifluoromethylphenyl)-5-trifluoromethyl-2-[³H]benzimidazolone; 1-EBIO: 1-ethyl-2-benzimidazolinone; DCEBIO: 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one; SKA-31: naphtho[1,2-d]thiazol-2-ylamine; NS309: 6,7-dichloro-1H-indole-2,3-dione 3-oxime; CyPPA: cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)6-methylpyrimidin-4-yl]-amine; GW542573X: 4-(2-methoxy-phenylcarbamoyloxymethyl)-piperidine-1-carboxylic acid tert-butyl ester; NS11757: N-(6,7-dichloro-1,2,3,4-tetrahydronaphthalen-1-yl)-1H-1,3-benzodiazol-2-amine; CM-TPMF: N-{7-[1-(4-chloro-2-methylphenoxy)ethyl]-[1,2,4]triazolo[1,5-a]pyrimidin-2-yl]-N'-methoxy-formamidinium; B-TPMF: N-{7-[1-(4-tert-butyl-phenoxy-ethyl)-[1,2,4]triazolo[1,5-a]pyrimidin-2-yl]-N'-methoxy-formamidinium; NS8593: (R)-N-(benzimidazol-2-yl)-1,2,3,4-tetrahydro-1-naphthylamine; ICA-17043: 4-Fluoro- α -(4-fluorophenyl)- α -phenylbenzeneacetamide; UCL-1684: 6,10-diaza-3(1,3,8)(1,4)-dibenzena-1,5(1,4)-diquinolincyclodecaphane; E-4031: N-[4-[1-[2-(6-Methylpyridin-2-yl)ethyl]piperidine-4-carbonyl]phenyl].

potentially being useful in the treatment of Parkinson's disease and depression [19]. Finally, KCa2.2 knockout mice lacked the mAHP in hippocampal neurons [20]. However, overexpression of KCa2.2 reduced synaptic plasticity and demonstrated reduced hippocampal memory and learning [21], suggesting that selective blockers of KCa2.2 might enhance cognitive memory performance in dementia such as Alzheimer's [19]. We would like to point out that a subsequent increase or decrease in channel function, be it via a classic gating effect or by altering the number of channels in the plasma membrane would be expected to have the same outcome in that it would decrease total current flow.

An additional condition in which KCa2.x channels have been implicated is detrusor overactivity (DO), which is a significant clinical problem having profound effects on the quality of life [22]. KCa2.x channels are important modulators of smooth muscle excitability and contractility of the urinary bladder [23] and underline the AHP in urinary bladder smooth muscle cells [24]. Nelson and coworkers demonstrated that suppression of KCa2.3 expression was associated with an increased frequency of phasic urinary bladder smooth muscle contraction *in vitro* and bladder overactivity *in vivo* [23]. Recently, Bouchelouche and colleagues demonstrated that the KCa2.x and KCa3.1 opener, NS4591 inhibited spontaneous contractile activity of human detrusor muscle [25]. Therefore, KCa2.x and KCa3.1 channels are potential targets for therapeutic management of urinary dysfunction related to DO and incontinence.

Finally, we would point out that the KCa2.x channels have been implicated in numerous additional physiological processes, including endothelial function which is briefly discussed below. However, it is beyond the scope of this review to cover these in detail and thus we would point the reader to several excellent reviews that have been recently published [1b, 4, 11, 26].

Intermediate-conductance KCa Channel (KCa3.1)

In 1937, Erickson reported that the K^+ concentration of sickle cells was 10% less than in normal red blood cells (RBC) [27]. Further, Tosteson et al. reported an imbalance in the cation concentrations of sickle cells that led to the description of dehydrated RBC with elevated levels of hemoglobin [28]. Subsequently, Gardos [29] described a Ca^{2+} -dependent loss of K^+ from RBC that was referred to as the 'Gardos Pathway'; later coined the 'Gardos Channel'. The molecular identity of the Gardos channel was determined nearly 30 years later by two separate groups [30]. This channel exhibits 42–44% identity and 50–55% similarity with the KCa2.x channels and was alternately named IK1 or SK4 and subsequently KCa3.1 (Table 1). Hoffman and colleagues later confirmed the presence of KCa3.1 in human RBC, firmly establishing this as the Gardos channel [31]. Identical to KCa2.x channels, KCa3.1 is composed of six transmembrane domains (S1–S6), with the pore loop located between S5 and S6, as well as cytoplasmic N- and C-termini [30, 32] (Fig. 1). KCa3.1 shares the CMBD in the proximal C-terminus with the KCa2.x channels and is similarly gated by sub-micromolar levels of cytosolic Ca^{2+} [6a, 6b]. KCa3.1 is also a weak inward rectifying K^+ channel, albeit with a larger conductance of 10–40 pS ($\pm$ 100 mV) [30, 32], hence the historical moniker of being an intermediate conductance channel. The intermediate conductance KCa channels have been historically distinguished from the KCa2.x channels in that they are blocked by both charybdotoxin and clotrimazole, but not apamin, whereas the KCa2.x channels are blocked by apamin while being insensitive to charybdotoxin and clotrimazole [30, 32a]. Recent advances in both opener and blocker pharmacology have dramatically advanced over the past several years, thereby allowing these channels to be further distinguished, as will be discussed below.

Regulation of KCa3.1

Similar to the KCa2.x channels, activation of KCa3.1 is dependent upon the constitutive binding of calmodulin to the conserved CMBD [6a]. Gerlach et al. first demonstrated a role for kinases in the regulation of KCa3.1 by showing that hydrolyzable ATP analogues, but not other nucleoside triphosphates, activated the channel via an increase in PoMAX without changing the apparent Ca^{2+} sensitivity; a method of activation distinct from the protein kinase CKII-dependent shift in Ca^{2+} -sensitivity observed for KCa2.2 [33]. Additionally, this ATP-dependent activation was shown to be mediated through PKA when expressed in *Xenopus* oocytes or when endogenously expressed in T84 cells, but was independent of PKA when heterologously expressed in HEK cells, suggesting additional kinases may also regulate KCa3.1. Using a series of KCa3.1/KCa2.3 chimeras, Gerlach et al. [34] further demonstrated that the kinase-dependent activation of KCa3.1 could be localized to a 14 amino acid domain within the C-terminus. Subsequently, Skolnik and colleagues utilized a yeast two-hybrid approach to identify the myotubularin-related protein 6 (MTMR6), a lipid (PI(3)P) phosphatase, as an interacting protein with a *C. elegans* KCa2.x family member [35] (Fig. 1). This interaction occurred via the channels coiled-coil domain, a region Syme et al. [36] had previously shown to be required for proper channel assembly and trafficking. Skolnik and colleagues [37] further demonstrated that this MTMR6-dependent regulation required the same 14 amino acid region of KCa3.1 previously shown to be required for ATP/kinase-dependent activation [34]. In an exciting next step, Srivastava et al. demonstrated that nucleoside diphosphate kinase B (NDPK-B), a histidine kinase, directly binds and activates KCa3.1 by phosphorylating His³⁵⁸ and that this is reversed by protein histidine phosphatase [38]. Further, NDPK-B knockout mice exhibit a reduced KCa3.1 activity [39], indicative of this pathway being functional *in vivo*, as well. An additional kinase which has been shown to regulate KCa3.1 function is PKC, although this appears to be an indirect effect as mutation of the four conserved PKC phosphorylation sites has no effect on activation [40]. Also, AMP-activated protein kinase (AMPK) has been shown to directly interact with the distal C-terminus of KCa3.1 (between Asp³⁸⁰ and Ala⁴⁰⁰), where activation of AMPK results in an inhibition of KCa3.1 function [41] and subsequent inhibition of transepithelial Cl^- secretion. As with KCa2.2, the total of these results suggest that a kinase/phosphatase protein regulatory complex exists for the careful regulation of KCa3.1 (Fig. 1).

Role of KCa3.1 channels in normal physiology and disease

KCa3.1 was originally identified in epithelial- and endothelial-rich tissues, including colon, lung, kidney, prostate, placenta and salivary gland, as well, as in the immune system where it is expressed in thymus, spleen, lymphocytes and bone marrow [30, 32]. More recently, KCa3.1 has also been identified within sensory neurons and microglia leading to the proposal that this channel may constitute a therapeutic target in the central nervous system for acute and chronic neurodegenerative disorders and treatment of secondary damage after spinal cord injury [42].

Within epithelia, KCa3.1 has long been known to be expressed in the basolateral (BL) membrane [43] where its activation by Ca^{2+} -mediated agonists results in an increased electrochemical driving force for Cl^- exit across the apical (AP) membrane, resulting in transepithelial Cl^- secretion. Activation of KCa3.1 is also known to modulate transepithelial Na^+ absorption by increasing the electrochemical driving force for Na^+ entry across the apical membrane [44]. Both ourselves [43a, 45] and others [46] have demonstrated that the activation of KCa3.1 by pharmacological modulators results in increased transepithelial Cl^- secretion across multiple epithelia leading to the proposal that pharmacological modulation of this channel may be therapeutically beneficial in diseases such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) [47], see below. Very recently, KCa3.1

expression in the BL membrane of colonic epithelia has been shown to be reduced 75% in patients with ulcerative colitis, which correlates with the reduced Na^+ absorption observed in these patients [48], suggesting that pharmacological upregulation of the remaining KCa3.1 channels may augment the reduced Na^+ absorption and be therapeutically beneficial. More recent reports have demonstrated that KCa3.1 may also be localized to the AP membrane of epithelial cells. That is, Rajendran and co-workers [49] described three distinct splice variants of KCa3.1 (KCNN4a , 425 amino acids; KCNN4b , 424 amino acids; and KCNN4c , 395 amino acids) and demonstrated that KCa3.1c was localized to the AP membrane while KCa3.1b was located within the BL membrane of rat colonic epithelial cells. KCa3.1a appears to be found in smooth muscle [49]. Further, Kumar et al. reported that agonist-dependent activation of KCa3.1c stimulated anion secretion in the rat distal colon and suggested that KCa3.1c may contribute to the stool K^+ losses associated with diarrheal illnesses [50]. Additional physiological roles of the splice variants of KCa3.1 will be unraveled by further investigations. Key additional questions concerning the trafficking of these unique splice variants to the BL and AP membranes remain, as discussed below.

As noted above, KCa3.1 plays a key role in RBC volume regulation [31, 51] as the loss of K^+ and Cl^- allows the RBC to shrink as it moves through the capillaries. This change in volume, and the resultant increase in hemoglobin concentration, dictates whether RBC will sickle, resulting in crisis in patients with sickle cell anemia. Thus, modulating KCa3.1 will regulate RBC volume and thus potentially be useful as a therapeutic target in this disease [52] (see below). KCa3.1 also plays a key role in T-cell function during the immune response as it traffics to the immunological synapse to regulate cell potential and thus Ca^{2+} entry [53]. Based on this, KCa3.1 has become a significant target for immunosuppression, as has been extensively reviewed by Wulff and colleagues [54].

KCa3.1 is co-expressed in vascular endothelia with KCa2.3 where these two channels play a key role in the endothelial derived hyperpolarizing factor (EDHF) response [55]. Indeed, knockout of either of these channels in mice results in an increase in mean arterial pressure [56] and this is further increased in animals in which both channels are knocked out [57]. Importantly, Köhler and colleagues [58] recently demonstrated that modulation of endothelial KCa3.1 and KCa2.3 resulted in a depressor response in a large conscious, unstressed dog model that resulted in functional and anatomical characteristics reminiscent of human cardiovascular function including changes in heart rate. These results further support the contention that pharmacological activation of KCa3.1 and KCa2.3 elicits vasodilation, and thus, a reduction in blood pressure *in vivo* by an EDHF-type vasodilator response. Therefore it is not surprising, that KCa3.1 and KCa2.3 are currently being evaluated as therapeutic targets for treatment of hypertension [55a, 59]. While both KCa3.1 and KCa2.3 are involved in the EDHF response they play unique roles, with KCa3.1 being critical to the acetylcholine-mediated vasodilation [60], whereas KCa2.3 is required during the sheer stress and reperfusion response [60]. For additional information, we would direct the reader to several recent, excellent reviews on the role of these channels in endothelial function [55a, 59]. Importantly for this review, KCa3.1 and KCa2.3 have been shown to target to unique sub-domains within endothelial cells, in that KCa3.1 targets to the region adjacent to the myoendothelial gap junctions, whereby it is capable of directly communicating the hyperpolarization to the underlying vascular smooth muscle, resulting in vascular relaxation [61]. In contrast, KCa2.3 targets to the caveolin-rich subdomains adjacent to the gap junctions between endothelial cells where they serve to propagate the hyperpolarizing response between endothelial cells [62]. These unique localizations are likely responsible for the differing physiological roles of these channels. However, the mechanisms by which these channels are correctly targeted to specific sub-domains and the mechanisms controlling their residence time in the plasma membrane, and hence their ability to regulate the response, are only now beginning to be elucidated, as detailed below.

Finally, we would point out that KCa3.1 has been implicated in numerous additional physiological processes, including cell proliferation and differentiation; leading to it being proposed as a target in cancer therapeutics [63], as well as a target for detrusor overactivity [64] and diabetes [65]. While it is beyond the scope of this review to cover these in detail, we would point the reader to excellent reviews which were recently published [66].

Pharmacological tools for KCa3.1 and KCa2.x

Pharmacological openers (activators)

As is apparent from the above discussion, KCa3.1 and KCa2.x channels play pivotal roles in a host of physiological processes and have been implicated in a wide array of disease pathologies. Early on, this led investigators in both academia and the pharmaceutical industry to search for both openers (activators) and blockers (inhibitors) of these channels based on the notion that they would be therapeutically useful in modulating the immune response (recently reviewed [54]), cellular proliferation [63, 67], smooth muscle tone and hence blood pressure (recently reviewed [57b, 59b]), volume regulation and hence RBC function [52a, 68] as well as transepithelial ion transport function [43a, 45, 47, 69]. Devor et al. [43a, 69a] identified the first pharmacological openers of endogenously expressed KCa3.1 in a search for stimulators of transepithelial Cl⁻ secretion. These investigators initially evaluated the NeuroSearch compounds, NS004 and NS1619, both of which had been shown to activate maxi-K⁺ channels [70]. Indeed, NS004 had also been shown to activate the cystic fibrosis transmembrane conductance regulator, CFTR [71]; the apical membrane Cl⁻ conductance in numerous secretory epithelia. However, neither NS004 nor NS1619 stimulated an appreciable Cl⁻ secretory response in the model T84 colonic epithelial cell line [69a]. In contrast, the structurally related compound, 1-EBIO (Fig. 2), which was identified by A.K. Singh while searching for existing compounds with the benzimidazolone backbone, induced a significant transepithelial Cl⁻ secretory response, having a half-maximal effective concentration of 490 μ M [43a, 69a]. It was further shown that this unique pharmacological profile was based on the ability of 1-EBIO to activate endogenous, basolateral KCa3.1, whereas the NeuroSearch compounds failed to modulate the activity of these channels [69a]. A further characterization of 1-EBIO demonstrated a direct, Ca²⁺-dependent activation of endogenous KCa3.1 [69a], suggesting that this basic chemical backbone could be further chemically manipulated to increase the affinity and specificity of KCa3.1 modulators. These initial observations were subsequently confirmed and extended on heterologously expressed KCa3.1, as well as the structurally similar KCa2.x channels by investigators at NeuroSearch [32b, 72], as well as ourselves [73].

Following these initial observations, we searched the Food and Drug Administration (FDA)-approved drug list to identify compounds structurally similar to 1-EBIO and evaluated these for activity against endogenously-expressed KCa3.1. In this regard, we identified chlorzoxazone (Parafon Forte DSC), a centrally acting smooth muscle relaxant, as well as the structurally similar zoxazolamine (2-amino-5-chlorzoxazole) as activators of KCa3.1 and further demonstrated that these compounds could stimulate transepithelial Cl⁻ secretion across primary cultures of human airway as well as a transepithelial potential difference response across nasal epithelium in normal human volunteers [47a]. These observations led us to propose that activators of KCa3.1 may be useful in diseases such as CF and COPD [47b, 69b]. These compounds, as well as the structurally similar riluzole (2-amino-6-trifluoromethoxy benzothiazole; Rilutek), were subsequently shown to activate heterologously expressed KCa3.1 as well as KCa2.x channels [73–74].

While each of the above compounds efficaciously activates KCa3.1 and KCa2.x channels they all suffer from being non-selective amongst the channel family members and also having relatively poor affinities, being ~50–500 μ M depending on whether activity was

assessed directly in excised patch-clamp experiments or in intact cell systems. In an attempt to identify higher affinity activators of KCa3.1, Bridges and colleagues [47b] carried out a structure activity study resulting in the first sub-micromolar affinity (840 nM) activator of KCa3.1, DCEBIO. More recently, Wulff and colleagues [75] used riluzole as a starting point for their medicinal chemistry efforts to synthesize SKA-31 which has an affinity of ~200 nM to 2 μ M, depending on which KCa family member was studied. Importantly, in a series of studies, investigators at NeuroSearch have identified compounds that display significantly higher affinities for KCa3.1 and KCa2.x activation, but perhaps more crucially some of these compounds also display KCa subtype specificity. Strøbæk et al. [76] characterized NS309 as a 10 nM affinity activator of KCa3.1 and KCa2.x channels, making this compound ~30-fold more potent than DCEBIO. More recently, Christophersen and colleagues characterized CyPPA as an activator of KCa2.2 and KCa2.3 channels, while having no effect on KCa2.1 and KCa3.1 [77], whereas GW542573X selectively activates KCa2.1 channels [78]. These studies elegantly demonstrate that selective modulators can be developed, enabling discrimination between KCa family members. However, additional work to evaluate their potential as pharmacological tools is required, given some lack of subtype specificity effects reported by the authors. As detailed in the references above, each of the compounds described has been shown to activate KCa2.x channels via a shift in the Ca^{2+} concentration-response curve to lower values such that the channels become active at resting levels of Ca^{2+} . However, as part of their drug development efforts, investigators at NeuroSearch have also identified compounds which act as negative modulators of KCa2.x family members [79], i.e., these compounds inhibit channel activity by shifting the Ca^{2+} affinity of the channel to higher values such that the channel becomes less active at any given Ca^{2+} concentration; a mode of action completely unique to typical channel inhibitors. The highest affinity negative modulator, NS11757, inhibited KCa2.3 currents with a IC_{50} of 9 nM [79]. Very recently, these studies were extended by identifying positive and negative modulators, CM-TPMF and B-TPMF, respectively, that are 10- to 20-fold more potent for KCa2.1 over other KCa2.x and KCa3.1 channels [80]. Indeed, the negative enantiomer of B-TPMF represents the first inhibitory compound with significant selectivity amongst the KCa2.x family members.

As noted, the openers and inhibitors detailed above work by shifting the apparent Ca^{2+} affinity of the KCa channels such that they display higher or lower open probabilities (P_o) at any given level of intracellular Ca^{2+} . Initially it was proposed, using 1-EBIO, that this shift in affinity was due to these compounds interacting at the calmodulin/CMBD of the channel in the proximal cytoplasmic C-terminus [81]. 1-EBIO significantly affected the Ca^{2+} -dependent deactivation of KCa3.1 while having little effect on the Ca^{2+} -dependent activation of the channel [81]. Given that deactivation occurs as a result of Ca^{2+} dissociating from CaM, it was concluded that 1-EBIO likely stabilized the Ca^{2+} -calmodulin-CMBD interaction [81]. As noted, until recently, high affinity, specific modulators of KCa function did not exist and thus more classic methods of identifying the binding site for these compounds were not available. Recently however, this roadblock has been overcome with the development of both high affinity and KCa subtype specific modulators, thereby allowing the binding site for these compounds to be re-evaluated. In preliminary data presented at the 2007 Biophysical Society Meeting (Long Beach, CA), Hougaard et al. [82] utilized the selective KCa2.3 modulator CyPPA in conjunction with KCa2.3/KCa2.1 chimeras to define the CyPPA binding domain as being within, and just distal to the CMBD, consistent with the site believed to be important for 1-EBIO activation. However, in an exciting series of recent studies, the focus has shifted away from the CMBD of these channels to the pore for both positive and negative modulation. In this regard, Christophersen and colleagues demonstrated, using a chimeric and mutagenesis approach, that both GW542573X- and B-TPMF-dependent modulation of channel activity depended upon Ser²⁹³ in the S5 segment of KCa2.1 [78, 80] (Fig. 3). However, as noted by these

authors, the structural differences in these compounds suggests that Ser²⁹³ may not represent part of the physical binding site, but rather this mutation may alter the allosteric coupling to the Ca²⁺-dependent activation. Indeed, in Fig. 3 this amino acid in S5 is buried within the protein rather than being exposed in the inner pore vestibule. In contrast, in an elegant study, Wulff and colleagues [83] used the negative gating modulator NS8593, which is specific for KCa_{2.x} channels over KCa_{3.1} channels, in combination with a series of KCa_{2.3}/KCa_{3.1} chimeras to demonstrate that the activation site for these compounds is in fact in the inner pore vestibule of the channel. Indeed, the amino acids identified correspond to Thr²⁵⁰ and Val²⁷⁵ in KCa_{3.1}; the amino acids previously shown to be required for block by clotrimazole, TRAM-34 [84] and also arachidonic acid as well as other fatty acids [85] (Fig. 3). While it may initially seem surprising that interaction of these compounds with amino acids in the pore vestibule would alter apparent Ca²⁺ affinity, it is worth noting that mutations in the pore lining S6 transmembrane domain alone, or in concert with cysteine-dependent modification, have been shown to cause a left-shift in the Ca²⁺-dependence of gating of KCa_{3.1} suggesting that these amino acids function to allosterically couple the energy of Ca²⁺ binding to the activation gate of this channel [86]. We have also identified mutations in S6 of KCa_{2.3} at Gly⁵³⁴ and Cys⁵³⁵ that cause a right-shift in the Ca²⁺-dependence of gating (D.C. Devor and M.A. Bailey, unpublished observations). Finally, Li and Aldrich identified charged amino acids in KCa_{2.2} at the bottom of S6 that, when mutated, shifted the apparent Ca²⁺ affinity for activation [87]. In total, these results lend further support to the notion that perturbations of these porelining amino acids by pharmacological compounds could similarly induce a shift in the Ca²⁺ concentration-response signature of these channels. Given that these activators and inhibitors function by altering the Ca²⁺-dependent gating of KCa_{2.x} and KCa_{3.1} channels it will be important in the future to determine whether the trafficking in to and out of the plasma membrane of these channels is similarly modified or whether the entire physiological effect of these compounds can be accounted for by changes in Po (see below).

To date, no KCa channel openers have been evaluated in clinical trials. However, Singh et al. [47a] demonstrated that the FDA-approved chlorzoxazone was capable of stimulating an *in vivo* transepithelial Cl⁻ secretory response in the airway epithelium of normal human volunteers by measuring an increase in the nasal transepithelial potential difference; consistent with activation of KCa_{3.1} in airway epithelia demonstrated in *in vitro* model systems [47, 69b]. Despite the paucity of human *in vivo* data, tantalizing glimpses of the potential to activate these channels in relevant model systems have been gleaned from studies carried out both in intact tissues and in *in vivo* animal models. Hamilton and colleagues [45] demonstrated that both 1-EBIO and DCEBIO are capable of stimulating transepithelial Cl⁻ secretion across intact mouse intestine; confirming the earlier studies on cell models and primary epithelial cultures, while Dong et al. [88] demonstrated that 1-EBIO stimulates duodenal bicarbonate transport *in vivo*. Further, Ferens et al. [89] demonstrated that DCEBIO modulated intestinal motility *in vivo*. Vick et al. [90] using 1-EBIO and CyPPA, concluded that KCa_{2.x} channel activation *in vivo* regulates the cellular mechanisms of memory encoding, while Allen et al. [91] reported that 1-EBIO reduced ischemia-induced CA1 neuronal cell death; improving cognitive outcome. In addition, *in vivo* perfusion of 1-EBIO in to the cerebellum of ataxic mice improved motor function [15a]. Further, 1-EBIO reduced seizure incidence in mice following electroshock, suggesting specific KCa openers may be useful as anticonvulsants [92]. Finally, numerous studies have shown that these compounds are capable of inducing endothelial hyperpolarization and subsequent vasorelaxation (recently reviewed [59b]) and very recently SKA-31 has been shown to induce endothelial hyperpolarization and decreased blood pressure in a conscious dog model [58]. While numerous additional examples exist to demonstrate the potential for KCa openers, these studies outlined suggest that further development of these positive modulators of KCa function may prove therapeutically beneficial. Clearly, additional work is needed for further

chemical optimization of subtype-selective KCa channels modulators, achievement of tissue specificity and elimination of off-target effects in whole organisms.

Pharmacological blockers (inhibitors)

Fortunately, for investigators, numerous blockers of the KCa3.1 channel have been developed, including both peptide toxins and small molecule inhibitors, thereby providing an impressive toolbox for the identification of the role of KCa3.1 in complex tissues. As these KCa3.1 channel blockers have been extensively reviewed in the recent literature [66b, 93] they will be discussed only briefly here. The antimycotic, clotrimazole was the first important small molecule blocker of KCa3.1 channels reported when Alvarez et al. [94] demonstrated, via either light scattering or isotopic flux assays, that clotrimazole inhibited a Ca^{2+} -dependent K^+ conductance in RBC. Rufo et al. [95] later confirmed these results in polarized colonic epithelial cells (T84 cells) using short-circuit current measurements combined with isotopic flux assays. Finally, while attempting to define the mechanism by which arachidonic acid inhibited endogenously expressed KCa3.1 [85b], Devor et al. [96] demonstrated a direct inhibition of KCa3.1 by clotrimazole in excised patch-clamp recordings. These early reports demonstrated that the imidazole ring of clotrimazole was not important to the demonstrated block of KCa3.1, indicating that a cytochrome P-450-like protein was not involved in the regulation of the channel [95–96]. Following these reports, it was demonstrated that clotrimazole could block cell shrinkage in RBCs [52a, 68], indicating KCa3.1 may be a viable therapeutic target in RBC sickling diseases. Based on this premise, Brugnara and colleagues, in collaboration with investigators at ICAgen, identified ICA-17043 (Senicapoc®) as a potent inhibitor (11 nM) of KCa3.1 and RBC dehydration [97]. Brugnara and colleagues have reported additional high affinity blockers of KCa3.1 channels in patent form (reviewed in [93]), although, to our knowledge, these compounds have not been published in the scientific literature. Wulff et al., [98] similarly used the clotrimazole backbone to synthesize TRAM-34, a high affinity blocker (20 nM) of KCa3.1 that has been extensively utilized to both define the role of these channels in normal physiology as well as explore their potential as therapeutic targets in disease (reviewed in [66b, 93]). Finally, investigators at Bayer demonstrated that the 4-phenyl-4H-pyrans [99] and the cyclohexadienes [100] are high affinity blockers of KCa3.1 activity with an IC_{50} of 1–10 nM being reported. While these blockers would appear to hold great promise, especially for RBC sickling diseases, clinical trials have so far proven disappointing [52b, 101].

In contrast to the relative wealth of KCa3.1 blockers, the toolbox for KCa2.x blockers is not as well stocked. More than 25 years ago, tubocurarine was shown to be a high affinity blocker of endogenously expressed KCa2.x channels [102]. Subsequently, dequalinium was shown to be a potent blocker (IC_{50} ~200–300 pM) of these channels both endogenously [103] and heterologously expressed [104]. Based on the dequalinium backbone, Galanakis et al. [105] undertook a synthetic chemistry tour-de-force which resulted in UCL-1684, a 200 pM inhibitor of KCa2.2 channels and which also has some specificity over other KCa2.x family members [104, 106]. While this molecule has been extensively utilized in the literature, additional small molecule blockers of KCa2.x channels have proven elusive (note that we discussed molecules such as NS8593 above and these inhibitors work by shifting the apparent Ca^{2+} affinity of the channel, rather than a more classical pore blocking mechanism). For a more thorough examination of the medicinal chemistry efforts that led to UCL-1684, we would direct the reader to the excellent review by Wulff et al. [66b].

Trafficking of KCa channels

The physiological response of ion channels depends critically on their number at the cell surface, which is determined jointly by anterograde and retrograde pathways. Recent studies

on the trafficking of KCa channels have revealed key molecular components in the regulation of assembly, delivery to the plasma membrane and subsequent internalization and degradation of these channels, opening the avenue for new therapeutic strategies that focus on the regulation of ion channel density at the cell surface. A schematic representation of the known intracellular trafficking pathways for KCa2.x and KCa3.1 are presented in Fig. 4.

Anterograde pathway

Prior to being engaged into the secretory pathway towards the plasma membrane (Fig. 4), KCa channels must be correctly folded and assembled into tetramers at the level of the endoplasmic reticulum (ER) [107]. During the past decade, several imaginative experimental approaches helped identify motifs or regions within the N- and C-termini of KCa channel subunits, which are critical for mediating proper folding and subunit interactions. Moreover, as discussed in the sections above, KCa channels are multiprotein complexes and the correct association of the channel with its regulatory components is vital for the ultimate targeting and biophysical properties of the channel.

Employing a series of carefully designed mutagenesis experiments, the groups of Kaczmarek [108] and Adelman [109] demonstrated that, apart from its role in channel gating as discussed above, constitutive association of calmodulin with the proximal C-terminal domain of KCa channels (CMBD) regulates the surface expression of KCa proteins by regulating the multimerization of channels through interactions between proximal C-terminal domains of monomers. Moreover, it has been shown that kinases and phosphatases are constitutively associated with KCa3.1 and KCa2.x (Fig. 1) and that this involves a close association between the N- and C-termini, that ultimately effects channel expression and function [10, 110].

Another important finding is that C-terminal coiled-coil domains within KCa3.1 and KCa2.x function in both channel assembly and in providing a protein scaffold with direct implications on cell surface expression. As mentioned above, initial studies by Khanna et al. identified a C-terminal leucine zipper, distal to the CMBD, as important for producing a hyperpolarizing K⁺ conductance [6b]. Devor and colleagues [36] further demonstrated that this conserved C-terminal leucine zipper is required for the correct targeting of the channel to the plasma membrane. Thus, while mutations of the leucine zipper do not compromise multimer formation, they do alter the assembly of the distal C-terminal tail of KCa3.1, affecting the proper folding of the channel. In addition, leucine zippers are known to be involved in protein-protein interactions [111]. Therefore, the C-terminal leucine zipper in KCa3.1 may be required for interactions with regulatory proteins important for channel trafficking to the plasma membrane. As detailed above, the PI(3)P phosphatase, MTMR6, interacts with KCa3.1 via the coiled-coil domains on both proteins, negatively regulating channel function by dephosphorylating PI(3)P in the membrane adjacent to KCa3.1 [35]. Whether similar interactions take place early on along the anterograde pathway that regulate the trafficking of the channel to the plasma membrane remains to be determined. In this case, these novel associations may represent important targets to be exploited in the development of pharmacological modulators of channel density at the cell surface. In contrast to KCa3.1, the folding function of the leucine zipper does not seem to be conserved in the KCa2.x family of channels, suggesting a clear divergence in the structural requirements for the folding and trafficking of KCa family members [36]. However, it has been shown by Chiamvimonvat and coworkers that direct physical interaction of the coiled-coil domains in the C-termini of KCa2.x channels are responsible for channel heteromultimer assembly [7b].

While initial studies focused on the role of the C-terminus in trafficking of KCa channels, more recent efforts have also divulged a role for the N-terminus in anterograde trafficking.

Using a combination of biochemical, immunofluorescence and patch-clamp techniques, Jones et al. demonstrated that the N-terminus of KCa3.1 contains overlapping leucine zipper (Leu¹⁸/Leu²⁵/Leu³²/Leu³⁹) and dileucine (Leu¹⁸/Leu¹⁹) motifs essential for channel tetramerization and correct trafficking to the plasma membrane [112]. The role of the N-terminus in KCa2.x channel subunit assembly was documented in a series of initial reports, as well [106] [113]. Thus, Fanger et al. [106] demonstrated that overexpression of an N-terminal fragment of KCa2.2 in Jurkat cells resulted in dominant-negative suppression of native channels via co-assembly of the truncated fragments with native subunits into non-functional tetramers, suggesting that KCa2.2 channels contain a tetramerization domain in the N-terminus. A similar dominant-negative effect was reported for an N-terminal fragment of KCa2.3, which resembles a rare truncation mutant of KCa2.3 identified in a patient with schizophrenia [113]. Additionally, Roncarati et al. [114] demonstrated that domains in both the N- and C-termini of KCa2.3 are required for channel expression at the plasma membrane, although the exact molecular motifs involved have not been defined. Thus, using truncated KCa2.3 channels, it has been shown that the N-terminus and the proximal C-terminus with the CMBD are important in the early trafficking step from the ER to the Golgi, whereas the distal C-terminus of KCa2.3 is required to exit the Golgi, possibly by interaction with vesicular carriers in the anterograde pathway [114].

Apart from the N- and C-termini, the critical role for a lysine residue located within the intracellular loop of KCa3.1 (Lys¹⁹⁷) and KCa2.3 (Lys⁴⁵³) in protein trafficking has been documented in a study by Jones et al. [115]. Mutations at these residues determined that the fully assembled channel was sequestered within the ER, suggesting that critical interactions with possibly other regulatory proteins, required for channel degradation and/or trafficking, are disrupted.

At present, there is a large gap in our understanding between early biogenesis and the interaction of KCa channels with either general chaperones or specific accessory factors which tightly regulate the quality control and exit from the ER of these channels. An important advance in our understanding of these processes came with the study of Devor and colleagues [116], demonstrating that charged amino acids in the S3 and S4 transmembrane domains of voltage-independent KCa channels play an evolutionary conserved role in channel biogenesis, similar to what has been reported for voltage-gated K⁺ channels [116]. Thus, the conserved glutamic acid in S3 and the two arginines in S4 of both KCa3.1 and KCa2.3 are critical determinants in establishing the electrostatic interactions required for the initial folding process and formation of salt bridges necessary to maintain the fundamental fold of the S1–S4 domain. This study further delineated, for the first time, the role of the ER integral membrane protein Derlin-1 and cytosolic chaperone AAA-ATPase p97 in ER translocation, protein dislocation and targeting of misfolded KCa channels for proteasomal degradation [116].

Plasma membrane localization

Once correctly synthesized and assembled, KCa channels are routed to the plasma membrane. Data obtained on a diversity of cell types and tissues indicate that there is a clear distinction in the sorting of KCa3.1 and KCa2.x channels into specific membrane microdomains or regions, and this may represent an additional regulatory mechanism by which these channels accomplish their physiological function. In this respect, it has been shown in endothelial cells that KCa2.3 channels reside within caveolin-rich lipid domains, while KCa3.1 are found in a different membrane compartment not associated with caveolin or lipid rafts [62]. A close association between KCa2.3 and caveolin-1 was documented by others [62] as well as in our recent studies (D.C. Devor, submitted manuscript).

KCa3.1 and KCa2.x are not only distinct with respect to their localization within membrane microdomains, but they also present a polarized distribution at the plasma membrane. Thus, as mentioned in the above section, an interesting immunohistochemical study on rat mesenteric artery endothelial cells reported a spatial separation of the two channels, with KCa3.1 present at the myoendothelial gap junctions, whereas KCa2.3 is localized at the adjacent endothelial cell gap junctions [61]. This distribution might emphasize the distinct contribution of these channel in these vascular beds, since KCa3.1 participates in the acetylcholine-mediated hyperpolarization subsequent to phenylephrine-mediated depolarization, whereas KCa2.3 has been implicated in the endothelium-dependent hyperpolarization of smooth muscle [60].

In T lymphocytes it has been shown that KCa3.1 is evenly distributed on the plasma membrane, but rapidly translocates to the immunological synapse upon antigen stimulation, as part of the signaling complex that facilitates T cell activation [53]. Additionally, it has been shown that KCa3.1 localizes with F-actin to the immunological synapse, emphasizing the role of the cytoskeleton in providing directionality for channel movement [53]. An interesting observation is that signaling molecules such as protein kinase A (PKA) and C (PKC), which are known regulators of KCa3.1 function, accumulate in the immunological synapse upon T cell activation, as well [117]. This close proximity could provide a regulatory mechanism for KCa3.1 function at the synapse. The polarized distribution of KCa3.1 channels has also been demonstrated in migrating Madin-Darby Canine Kidney (MDCK) cells, where KCa3.1 accumulates at the leading edge of the lamellipodium [118]. Moreover, as mentioned in the section above, there is targeted distribution of KCa3.1 in secretory epithelia, where KCa3.1 has long been known to be expressed at the BL membrane [43, 119]. However, recent data on colonic epithelial cells provides evidence for the additional presence of KCa3.1 in the AP membrane, and demonstrates that the AP and BL membrane localized channels represent unique splice variants of KCa3.1 [49]. Thus, KCa3.1c (lacking the predicted second transmembrane domain) and KCa3.1b are present in AP and BL membranes of colonic epithelial cells, respectively. Importantly, *in vitro* expression studies using oocytes demonstrate that KCa3.1b is readily localized at the plasma membrane, whereas KCa3.1c requires the BK (large conductance Ca^{2+} activated K^{+} channel) $\beta 1$ -subunit, which functions as a Ca^{2+} sensor for BK [120], as a chaperone for its expression at the cell surface [49]. As different KCa3.1 splice variants may regulate different cellular functions under physiological and pathophysiological conditions it is of great importance to elucidate each rate-limiting step in their polarized trafficking and function as a potential avenue for therapeutic intervention. In this respect, a recent study emphasizes the role of the N-terminus in the directional trafficking to the plasma membrane and function of KCa3.1, demonstrating that a new KCa3.1 isoform lacking the N-terminus is retained within the cytosol and acts as a dominant negative on the full length channel in human lymphoid tissues [121]. This is consistent with the previously published role of the N-terminus in KCa3.1 trafficking [112].

In neurons, the KCa2.x members also present a distinct localization within neuronal compartments, with KCa2.2 largely postsynaptic, whereas some KCa2.3 channels are present presynaptically, and this has fundamental implications on neuronal excitability and function [1b, 26a, 122]. Moreover, when expressed in hippocampal neurons, KCa2.1 produces functional channels only in the soma and proximal processes, and this was attributed to a C-terminal YxxxR motif [123]. As for KCa3.1, splice variants of KCa2.x partially or totally lacking the N-terminus have been described [7a, 12b], with dramatic effects on channel localization and function. Thus, two isoforms of KCa2.2 have been described with different lengths of the N-terminal domain: KCa2.2-L has 207 additional N-terminal amino acids compared with KCa2.2-S [7a]. It has been shown that KCa2.2-L expression is essential for KCa2.2 heterotetramer localization and is also required for normal synaptic responses,

plasticity and learning [124]. Additionally, an isoform of KCa2.3 lacking the N-terminus and the first transmembrane segment has been shown to have a dominant negative effect on endogenous KCa2.3 currents by altering the distribution of KCa2.3 proteins [12b].

From all these studies, it appears that the directed targeting of KCa channels to the plasma membrane and channel redistribution in response to different stimuli must be tightly regulated. The molecular determinants of these processes and how they work together are poorly understood at present. However, it is plausible to hypothesize that the specifics of these mechanisms may result from a concerted action of specialized trafficking and specific anchoring at the cell surface. Ongoing studies will help to gain both basic and clinical insight into KCa trafficking at the plasma membrane, offering the opportunity to identify new pharmacological targets for modulating KCa channel density.

Retrograde pathway

Compared to the extensive studies on the KCa channels anterograde trafficking, the mechanisms of endocytosis and downstream sorting of these channels is just beginning to be unraveled (Fig. 4). Recently, Devor and colleagues [125] developed a strategy for monitoring the endocytic routes of KCa3.1 and KCa2.3, based on the work of Ting et al [126]. This involved inserting a biotin ligase acceptor peptide (BLAP) sequence into the second extracellular loop of these channels, followed by a fast and specific biotinylation of the KCa channels at the cell surface using recombinant biotin ligase [125, 127]. Addition of (fluorophore conjugated) streptavidin allows the fate of the endocytosed channels to be addressed using a combination of biochemical and imaging techniques [125, 127]. Using this approach, it was demonstrated, in expression systems, that KCa2.3 has a long half-life at the plasma membrane (~ 13 hr) which could be attributed to a dynamic recycling of the channel back to the cell surface in an RME-1- and Rab35/EPI64C-dependent manner [125]. Importantly, the study identified a 12 amino acid motif within the N-terminus of KCa2.3 (Gly²⁰⁶-Pro²¹⁷) required for the association with RME-1 and hence the fast recycling of the channel [125]. Consistent with a caveolar localization of the channel as discussed above, Devor and coworkers demonstrated that KCa2.3 endocytosis is dynamin dependent and they documented the role of the small GTPase Rab5 in the formation of the KCa2.3 containing endocytic vesicles and subsequent delivery to the recycling endosomes (submitted manuscript).

The fast dynamics at the plasma membrane seems to be a general characteristic of KCa2.x channels, although some tissue-specific differences have been reported and this may reflect different trafficking in different cell types (see below). Thus, postsynaptic KCa2 channels (most likely KCa2.2) in lateral amygdala pyramidal neurons also undergo constitutive recycling and dynamin-dependent endocytosis [128]. In addition, actin plays an important role in channel stability at the plasma membrane and in its recycling back to the synapse. More importantly, as detailed in a series of initial studies and recently reviewed [1b], kinases are directly involved in KCa channel trafficking. Thus, activation of β 1 adrenoceptors in amygdala pyramidal neurons triggers activation of the PKA pathway, which interferes with KCa2.2 recycling, effectively sequestering the channels into the cytosolic compartment [128]. This translates into an enhanced excitatory synaptic transmission and plasticity. On the other hand, in CA1 pyramidal neurons the synaptic KCa2.2 channels do not constitutively recycle and they are internalized after chemically or physiologically relevant induction of long-term potentiation (LTP), with this process also involving channel phosphorylation by PKA [129]. Interestingly, the trafficking of KCa2.2 channels is coordinately regulated with spine AMPA receptor trafficking. Thus, upon the induction of LTP, the rapid exocytosis of AMPA receptor triggers the endocytosis of synaptic KCa2.2 channels [130].

In contrast to its family members, KCa3.1 does not seem to share the same fate at the plasma membrane. Using the BLAP-tag methodology described above, Balut et al. [127] demonstrated that KCa3.1 has a much shorter half life at the cell surface, being completely internalized within 60–90 min (Fig. 4). This rapid endocytosis, suggested to be clathrin-dependent, was confirmed by Schwab et al. in MDCK migrating cells [131]. In addition, a dileucine motif present in the C-terminus of KCa3.1 (Leu³⁴⁴/Leu³⁴⁵) appears to be important for channel internalization [131]. Subsequent to endocytosis, KCa3.1 is targeted for lysosomal degradation via a Rab7-, and MVB (multivesicular body)/ESCRT (endosomal sorting complex required for transport)-dependent pathway [127]. By combining BLAP-tagged KCa3.1 with TUBEs (tandem ubiquitin binding entities) and DUB Chip methodologies, Balut et al. [132] further demonstrated that poly-ubiquitylation mediates the targeting of internalized KCa3.1 to the lysosomes and also that USP8 regulates the rate of KCa3.1 degradation by deubiquitylating KCa3.1 prior to lysosomal delivery. Importantly, it has been shown that KCa3.1 endocytosis can be reduced by blocking the ubiquitin activating enzyme E1 [132–133], suggesting that ubiquitylation of either the channel itself or associated adaptors functions as a signal for KCa3.1 endocytosis. The plausibility of this pathway is supported by recent data showing that in CD4 T cells the tripartite motif containing protein 27 (TRIM27)-dependent ubiquitylation of the class II PI3K-C2 β kinase acts as a negative regulator of KCa3.1 activation [134]. Thus, TRIM27 functions as an E3 ligase and mediates lysine 48 polyubiquitylation of PI3K-C2 β kinase, leading to an inhibition of the PI3K enzyme activity. This leads to decreased levels of PI(3)P which, as discussed above, results in a decreased histidine phosphorylation and activation of KCa3.1 by NDPK-B [134].

KCa Trafficking as a Therapeutic Target

As summarized in this review, studies from the last decade, as well as ongoing research, provide a growing understanding of the mechanisms involved in KCa channel assembly and their recruitment into multiprotein complexes, thereby controlling their intracellular trafficking routes and stability at the plasma membrane. The physiological contribution of KCa channels is ultimately determined by the number of functional channels and their life time at the cell surface, and hence one new therapeutic avenue emerging is the extrinsic manipulation of KCa trafficking events, in order to control the number of channels at the plasma membrane.

A correlation between a disease state and the density of plasma membrane KCa3.1 channels has been recently addressed in a study showing that KCa3.1 expression and activity in the basolateral membrane of colonocytes is decreased in the pathogenesis of diarrhoea in ulcerative colitis [48]. Interestingly, KCa3.1 mRNA levels were not different between the normal and ulcerative colitis patients, leaving open the question that possibly altered translation or impaired anterograde trafficking of KCa3.1 might be determinants of this disease, and therefore targets for future therapeutic approaches. In line with this, molecules which correct the expression at the plasma membrane of mutant forms of CFTR [135] and hERG [136] have recently been described. Thus, glafenine, an anthranilic acid derivative with analgesic properties, administered at clinically relevant concentrations, was able to partially correct the mislocalization of the mutant Δ F508-CFTR and increase its functional expression at the cell surface [135]. Additionally, treatment with antiarrhythmic drugs, such as E-4031, rescues the trafficking deficit for nearly 70% of the mutant hERG channels by stabilizing the misfolded proteins [137]. However, it was suggested that this rescue occurs mainly by facilitation of quality control mechanisms, rather than directly affecting the trafficking machinery. Addressing these issues, recent studies have focused on the pharmacological regulation of the voltage-gated potassium channel, Kv1.5 stability within the membrane as well as their trafficking [138]. Thus, it has been shown that the antiarrhythmic drug quinidine, which blocks Kv1.5, can also trigger channel internalization

in a dose- and time-dependent manner ^[139]. Moreover, this internalization is isoform specific, since the drug can block Kv4.2 and Kv2.1 but has no effect on the trafficking of these channels. Importantly, the treatment with quinine, which is a diastereomer of quinidine, blocked Kv1.5 current but did not enhance channel internalization, suggesting that pore block is separable from drug induced trafficking events and that optimal drug design might allow for the manipulation of trafficking pathway ^[138]. In line with these findings, a recent study demonstrated that treatment with 1-EBIO prevented the endocytosis of KCa2.2 in synapses, with beneficial effects on neuronal survival and cognitive outcome following ischemic conditions ^[91, 124].

As discussed above, KCa channels exist as constitutive multiprotein complexes, with their associated regulatory proteins influencing multiple aspects of KCa physiology, including gating, pharmacology and plasma membrane expression. It is therefore tempting to consider the possibility of pharmacologically targeting these components or the domain-domain interactions they form with KCa channels, as a future beneficial clinical tool in diseases related to the expression and stability of these channels at the plasma membrane. Moreover, identifying the specific components regulating the trafficking of KCa channels, i.e., chaperones, small molecular weight GTPases and other regulatory elements, can be relevant in designing targeted therapeutics aimed to control channel surface density with reduced possible side effects.

Another promising area of investigation is ubiquitylation, which has been reported as a key player in regulating the trafficking and stability of a multitude of ion channels linked to several disease states ^[140]. As the role of this posttranslational modification in regulating KCa function starts to be unraveled, one can envision that development of pharmacologic modulators of the components of the ubiquitylation/deubiquitylation cascade may serve as novel therapeutic modalities for these channels. An excellent candidate in this respect is KCa2.3 which, as discussed above, exhibits rapid recycling kinetics at the plasma membrane, possibly regulated by a dynamic ubiquitylation/deubiquitylation of the channel, as shown for other recycling proteins ^[141]. Elucidating the components involved in this process, and their sites of interaction, represent challenging questions, and innovative methodologies and tools are beginning to emerge to permit these unanswered questions to be addressed. One main challenge to overcome is identifying the specific E3 ligase involved in this process. This is technically difficult due to the transient nature of the enzyme-substrate interaction and also the limited information on the various partnering motifs.

Of course, there are many open questions to be addressed in the study of KCa channels trafficking. What are the chaperones and accessory proteins that facilitate ER exit of KCa channels? Do members of the KCa family exit the Golgi and traffic to the plasma membrane via different routes and how is this regulated? What are the scaffolding proteins and accessory subunits involved in directed targeting of these channels to specific microdomains within the plasma membrane? How are these interactions regulated? These are all unsolved problems, opening new exciting directions to be explored. Deciphering the molecular mechanisms of KCa trafficking events is expected to bring into focus potential candidates for drug therapies with beneficial effects in a multitude of diseases related to these channels.

Outlook

KCa channels play a critical role in the physiology of a wide variety of tissues and disease states. Unfortunately, although research efforts during the last decade have resulted in the development of a multitude of drugs (activators and inhibitors) which can modulate the function of KCa channels positive therapeutic outcomes have not yet been realized. However, to date medicinal chemistry efforts which focus on the trafficking events that

ultimately determine the number of functional channels present at the cell surface have not been carried out, nor have the existing compounds been evaluated for their effects on these pathways. Tissue targeting and drug delivery to specific subcellular compartments are critical aspects in designing effective drugs to modulate ion channel trafficking events. As recently summarized by Simmons and colleagues ^[142], novel approaches for drug design, including trying to use specific sorting domains which enable the delivery of the drug to a specific intracellular location have recently been proposed to address these challenging issues. Therefore, insights into the anterograde and retrograde trafficking pathways as well as the mechanisms by which KCa channels are recruited into functional multiprotein complexes are crucial to our understanding of how channel density at the plasma membrane is maintained and thus potentially manipulated for therapeutic gain. Thus, these aspects of the cell biology of KCa channels represent important targets to be exploited in the near future by both basic and medicinal chemistry research. Indeed, pharmaceutical compounds could be developed to modulate the interaction of KCa channels with known regulatory proteins, or with proteins that are yet to be discovered, allowing for a targeted control of the number and residency time of the functional channels at the plasma membrane. This represents a new and promising direction for the development of new pharmaceutical modulators of KCa channels, with important therapeutic benefits.

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Figure 1. Schematic of KCa3.1 and KCa2.2 subunits, emphasizing the interaction with regulatory subunits, as described in the text. Both KCa3.1 and KCa2.2 constitutively bind calmodulin (CAM). See text for abbreviations.

Figure 2.
Chemical structures of KCa2.x and KCa3.1 channel activators and negative modulators, as described in the text.

Figure 3.

Homology model of the S5-Pore-Helix-S6 region for the open state of KCa3.1 using the rKv1.2 structure. Amino acids shown to be involved in pharmacological activation/inhibition of KCa2.x/KCa3.1 channels are highlighted, including Thr²⁵⁰ in the pore, Val²⁷⁵ in S6 and Leu²¹⁵ in S5 (Ser²⁹³ in KCa2.1). **Left panel.** Representation from the extracellular entrance showing all four subunits of the tetramer. **Right panel.** Profile view with only two of the four subunits shown for clarity. K⁺ ions shown in red.

Figure 4.

Schematic representation of anterograde and retrograde trafficking pathways for KCa2.x (left) and KCa3.1 (right) channels. The important motifs and regulatory proteins in channel assembly are illustrated. The molecular determinants of the forward trafficking of KCa channels are not fully determined at this time. Once at the plasma membrane, KCa2.x channels reside in caveolae. KCa2.3 is endocytosed in a dynamin and Rab5-dependent manner, after which KCa2.3 enters a recycling endosome (RE) compartment and is returned to the plasma membrane in a Rab35/EPI64C/RME-1 dependent manner. In contrast, KCa3.1 undergoes clathrin-mediated endocytosis and is rapidly targeted for lysosomal degradation via an ESCRT- and Rab7- dependent pathway. Ubiquitylation (Ub) mediates the targeting of membrane KCa3.1 to the lysosomes and USP8 regulates the rate of KCa3.1 degradation by deubiquitylating KCa3.1 prior to lysosomal delivery. CAM=calmodulin; MVB=multivesicular bodies. See text for additional details.

Table 1

Nomenclature of the KCNN gene family based on the HGNC and IUPHAR designations and chromosome location.^[a]

HGNC	IUPHAR	Common Names	Chromosome
KCNN1	KCa2.1	SKCa1, hSK1, SK1	19p13.1
KCNN2	KCa2.2	SKCa2, hSK2, SK2	5q22.3
KCNN3	KCa2.3	SKCa3, hSK3, SK3	1q21.3
KCNN4	KCa3.1	SK4, IKCa, IK1, Gardos channel	19q13.2

^[a] Official nomenclatures designated by the HGNC (HUGO Gene Nomenclature Committee) and IUPHAR (International Union of Basic and Clinical Pharmacology ^[3]) organizations. Modified from Girault et al. ^[4].