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Serine/Threonine Phosphatases in Atrial Fibrillation¹

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Abstract

Serine/threonine protein phosphatases control dephosphorylation of numerous cardiac proteins, including a variety of ion channels and calcium-handling proteins, thereby providing precise post-translational regulation of cardiac electrophysiology and function. Accordingly, dysfunction of this regulation can contribute to the initiation, maintenance and progression of cardiac arrhythmias. Atrial fibrillation (AF) is the most common heart rhythm disorder and is characterized by electrical, autonomic, calcium-handling, contractile, and structural remodeling, which include, among other things, changes in the phosphorylation status of a wide range of proteins. Here, we review AF-associated alterations in the phosphorylation of atrial ion channels, calcium-handling and contractile proteins, and their role in AF-pathophysiology. We highlight the mechanisms controlling the phosphorylation of these proteins and focus on the role of altered dephosphorylation via local type-1, type-2A and type-2B phosphatases (PP1, PP2A, and PP2B, also known as calcineurin, respectively). Finally, we discuss the challenges for phosphatase research, potential therapeutic significance of altered phosphatase-mediated protein dephosphorylation in AF, as well as future directions.

¹Abbreviations: AF: atrial fibrillation; AP: action potential; β -AR: β -adrenoceptor; cAF: chronic AF; CaMKII: Ca²⁺/calmodulin-dependent protein kinase type-II; cMyBP-C: cardiac myosin-binding protein-C; Cn: calcineurin; DAD: delayed afterdepolarization; HF: heart failure; IC_{Ca,L}: L-type Ca²⁺-current; I_{K1}: basal inward-rectifier K⁺-current; I_{K2P}: two-pore domain K⁺-current; I_{K,ACh}: acetylcholine-dependent inward-rectifier K⁺-current; I_{Kr}: rapid delayed-rectifier K⁺-current; I_{Ks}: slow delayed-rectifier K⁺-current; I_{Kur}: ultra-rapid delayed-rectifier K⁺-current; I_{Na}: Na⁺-current; I_{to}: transient-outward K⁺-current; NCX1: Na⁺-Ca²⁺-exchanger; pAF: paroxysmal AF; PKA: protein kinase-A; PKC: protein kinase-C; PLB: phospholamban; PP1: protein phosphatase type-1; PP2A: protein phosphatase type-2A; RyR2: ryanodine receptor type-2; SERCA2a: sarcoplasmic reticulum Ca²⁺-ATPase; SR: sarcoplasmic reticulum; TnI: troponin-I;

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Conflicts of Interest

X.H.T.W. is a founding partner of Elex Biotech, a start-up company that developed drug molecules that target ryanodine receptors for the treatment of cardiac arrhythmia disorders. D.D. is consultant for OMEICOS Therapeutics that develops drug molecules targeting the ω -fatty acid metabolism as an antiarrhythmic therapeutic strategy. J.H. and S.G. declared no conflicts of interest.

Keywords

protein phosphatases; atrial fibrillation; ion channels; calcium handling; myofilaments

1. Introduction

1.1. Atrial fibrillation

Atrial fibrillation (AF) significantly affects morbidity and mortality as a risk factor for worsening heart failure (HF) and stroke [1–3]. AF affects more than 33 million people worldwide, with an incidence that is expected to increase further as the population ages [4]. A large number of cardiovascular and non-cardiovascular diseases, as well as AF itself, increase the risk of AF by promoting proarrhythmic atrial remodeling, giving rise to a progressive atrial cardiomyopathy [1,5,6]. Clinically, this progression of AF is reflected by its classification into paroxysmal AF (pAF), persistent AF or long-standing persistent (chronic) AF (cAF), depending on the duration of the arrhythmia [3].

Conceptually, abnormal ectopic (triggered) activity and reentry are the main arrhythmogenic mechanisms underlying AF [2,7,8] (Figure 1). Atrial ectopic activity is predominantly promoted by (sub)cellular Ca^{2+} -handling abnormalities resulting in delayed afterdepolarizations (DADs) that may trigger atrial action potentials (AP) independent of the normal atrial activation. Sustained high-frequency ectopic activity can maintain AF through fibrillatory conduction [7]. In addition, ectopic activity can initiate reentry in a vulnerable substrate characterized by a short refractory period and slow and/or heterogeneous conduction [7]. Clinical AF is likely maintained through a combination of ectopic and reentrant mechanisms, with significant inter-patient variability in the exact balance between both mechanisms [2,9].

At the molecular level, atrial remodeling is mediated by various pathways [2,10,11]. Dysfunctional post-translational regulation through reversible protein phosphorylation may contribute both to the acute initiation of AF and to the substrate promoting AF maintenance [12,13] (Figure 1). The majority of all proteins in human beings undergo reversible phosphorylation, predominantly at serine or threonine residues [14,15]. Most proteins in the heart have multiple phosphorylation sites and the phosphorylation state of each site depends on a dynamic local balance between multiple kinases and phosphatases organized as holoenzymes in macromolecular complexes [16]. Combined, the phosphorylation state of all sites determines the function of each holoenzyme and allows precise control of atrial electrophysiology and contractility.

Type-1 phosphatases (PP1) and type-2 phosphatases (PP2), notably PP2A and PP2B (calcineurin; Cn) are the best characterized phosphatases [17] and make up the majority of phosphatase activity in the heart [18]. The catalytic subunits of these phosphatases are organized in multi-protein complexes with different regulatory and anchoring subunits that target the holoenzyme to specific subcellular locations to ensure precise local regulation of individual targets. This review will focus on the regulation of atrial electrophysiology, Ca^{2+} handling and contractility through PP1, PP2A and calcineurin and their potential roles in AF.

1.2. Cellular electrophysiology and excitation-contraction coupling in the atrium

For a detailed overview of cardiac cellular electrophysiology and excitation-contraction coupling, the interested reader is referred to [19–21]. Similar to ventricular cardiomyocytes, the upstroke of the atrial AP is mediated by an inward Na^+ -current (I_{Na}). Subsequently, the L-type Ca^{2+} -channel is rapidly activated, resulting in an additional inward current ($I_{\text{Ca,L}}$). The Ca^{2+} entering the atrial cardiomyocyte via the L-type Ca^{2+} -channels triggers a much larger Ca^{2+} release from the sarcoplasmic reticulum (SR) through type-2 ryanodine receptor (RyR2) channels. The total increase in cytosolic Ca^{2+} gives rise to the systolic Ca^{2+} -transient and initiates cardiomyocyte contraction. Repolarization is mediated by the voltage- and time-dependent activation of atrial K^+ -currents, including the transient-outward current (I_{to}), the ultra-rapid, rapid and slow delayed-rectifier K^+ -currents (I_{Kur} , I_{Kr} , and I_{Ks} , respectively), two-pore K^+ -current (I_{K2P}) and inward-rectifier K^+ -current (I_{K1}). The acetylcholine-activated inward-rectifier K^+ -current ($I_{\text{K,ACh}}$) furthermore contributes to faster repolarization during increased parasympathetic tone. Relaxation of atrial cardiomyocytes occurs when cytosolic Ca^{2+} levels decline due to reuptake of Ca^{2+} into the SR by the SR Ca^{2+} -ATPase type-2a (SERCA2a) and extrusion from the cell by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger type-1 (NCX1), with a small additional contribution from the plasmalemmal Ca^{2+} -ATPase. Finally, homeostasis of Na^+ and K^+ is achieved through the Na^+/K^+ -ATPase. Virtually all of these channels/transporters or their regulatory subunits can be modulated through reversible phosphorylation by protein kinases, notably protein kinase-A (PKA), protein kinase-C (PKC), and Ca^{2+} /calmodulin-dependent protein kinase-II (CaMKII), and dephosphorylation by PP1, PP2A and, to a lesser extent, Cn.

Despite the overall similarities between atrial and ventricular electrophysiology and Ca^{2+} -handling, there are important electrophysiological differences between both chambers. Several ion currents (I_{Kur} , I_{K2P} and $I_{\text{K,ACh}}$), are almost exclusively present in the atria, resulting in a more triangular AP morphology compared to ventricular cardiomyocytes [20]. These channels are currently explored as targets for new antiarrhythmic drugs with atrial-specific effects [8,22]. Similarly, the inhibitory SERCA2a-regulator sarcolipin is predominantly expressed in the atria [23] and atrial cardiomyocytes generally have a less well-developed system of transverse membrane invaginations (T-tubules) that bring L-type Ca^{2+} -channels into close proximity of RyR2 located towards the center of the cardiomyocyte [24], modulating the atrial Ca^{2+} -transient. Protein phosphatases are also differentially expressed between atria and ventricles, with increased PP1 and PP2A mRNA levels and increased PP2A, but unchanged PP1 protein expression in the right ventricle compared to the right atrium [25]. This difference may affect the basal phosphorylation state of atrial versus ventricular ion channels, thereby contributing to atrial/ventricular electrophysiological differences [26].

2. Remodeling of phosphatase activity and expression in AF

2.1 PP1

PP1 is ubiquitously expressed in most cell types, including atrial cardiomyocytes. Table 1 summarizes the alterations in PP1 in different AF paradigms. The global expression of PP1 is unchanged in cAF patients compared to sinus rhythm controls [27,28], but its enzymatic

activity is increased [29]. In pAF patients, PP1 catalytic subunit expression is borderline-significantly increased or unchanged [30,31]. The dog models of atrial tachycardia remodeling (ATR) and ventricular tachypacing-induced congestive HF (CHF), well-known experimental models with increased susceptibility to AF [32], show qualitatively similar PP1 alterations, despite the important differences in atrial remodeling between both models [33]. Taken together, these data suggest that increased PP1 activity is a typical finding for conditions associated with increased risk of AF. Three isoforms of catalytic PP1 subunits (PP1 α /PPP1CA, PP1 γ /PPP1CC, and PP1 δ also known as PP1 β /PPP1CB) have been identified [34] (see Supplementary Table 1 for an overview of protein phosphatase nomenclature). The mRNA levels of all three isoforms are either unchanged or reduced in cAF patients compared to sinus rhythm patients [28]. The absence of a clear increase in the expression of PP1 catalytic subunits in cAF patients, as well as different animal models, despite the increase in PP1 activity (Table 1), points to post-translational regulation of PP1 activity in AF.

PP1 catalytic subunits form dimers with numerous regulatory subunits that either target the holoenzyme to specific substrates or regulate its enzymatic activity [12,17]. The composition and role of the PP1 interactome in cardiovascular disease is reviewed in detail in [35], but there is only limited information available about AF-associated remodeling of PP1 regulatory subunits (Table 1). One notable exception to this is the regulatory subunit I-1 (PPP1R1A), a selective and potent PP1 inhibitor that facilitates crosstalk between different protein phosphatases and kinases [36,37]. PKA-dependent phosphorylation of Thr35 activates I-1. As such, PKA-dependent I-1 activation and subsequent PP1 inhibition form a positive feedback loop amplifying the phosphorylation of several substrates during β -adrenoceptor (β -AR) stimulation. Dephosphorylation of I-1 Thr35 is mediated by PP2A and Cn, thereby creating additional crosstalk between different phosphatases [38]. I-1 Thr35-phosphorylation levels are strongly increased (~10 fold) in cAF patients [39], likely resulting in local reductions in PP1 activity. Furthermore, PKC α phosphorylates I-1 on Ser67, decreasing I-1 activity and substrate phosphorylation, but Ser67 phosphorylation levels are unchanged in cAF patients [39]. Thus, AF-related changes in I-1-mediated regulation (inhibition) of PP1 are unlikely to explain the increase in global PP1 activity, but may produce local reductions in PP1 activity contributing to AF-associated hyperphosphorylation of specific targets, as discussed below.

2.2 PP2A

Similar to PP1, PP2A is ubiquitously expressed [40] and its activity is increased in cAF vs. sinus rhythm patients, although the magnitude of this increase appears smaller than for PP1 (Table 1). PP2A can exist as a dimer of a catalytic (PP2A-C) and scaffold (PP2A-A) subunits, or as a trimer of scaffolding, catalytic and regulatory subunits. PP2A-A (PPP2R1) expression is unchanged in cAF patients, but the expression of PP2A-C (PPP2C) is increased, potentially explaining the increased enzymatic activity [28]. By contrast, PP2A-C expression is unchanged in pAF patients [31]. Immunostaining of PP2A-A and PP2A-C can be observed throughout the cardiomyocyte, whereas regulatory subunits have specific subcellular localizations, for example B56 ϵ (PP2R5E) and PR72/PR130 (PPP2R3A) near the Z-line, or B56 γ (PP2R5C) and PR53 (PP2R4) near the nucleus, indicating that

functional regulation by PP2A is spatially-heterogeneous [40]. Regulatory subunits may also play a critical inhibitory role in the regulation of PP2A activity in the heart. For example, B56 α (PPP2R5A) overexpression decreases phosphatase activity and increases RyR2 phosphorylation, whereas loss of B56 α results in PP2A-mediated RyR2 dephosphorylation [41]. However, there is limited information about remodeling of PP2A regulatory subunit expression or distribution in AF, particularly in pAF patients.

Further regulation of PP2A activity can occur through phosphorylation of PP2A-C on Tyr307, which destabilizes the interaction with the regulatory subunit and reduces phosphatase activity, or methylation of Leu309, which increases activity [40]. Both modifications are promoted by conditions of oxidative stress [40], but despite the evidence for increased oxidative stress in AF [42], PP2A-C methylation levels between cAF and sinus rhythm patients are similar [39].

2.3 PP2B (Calcineurin)

Calcineurin (PP2B, Cn) is a Ca²⁺-dependent phosphatase consisting of catalytic (CnA) and regulatory (CnB) subunits that link Ca²⁺- and phosphorylation-dependent signaling pathways [15]. Three different CnA isoforms (CnA α /PPP3CA, CnA β /PPP3CB, and CnA γ /PPP3CC) have been identified, of which CnA α and CnA β are ubiquitously expressed. CnB is expressed by two genes (CnB α /PPP3R1 and CnB β /PPP3R2) [34]. Both expression and activity of CnA are increased in pAF [43] and cAF patients [44] as well as in cardiomyocytes paced at 3 Hz compared to those paced at 1-Hz [44,45] (Table 1), suggesting that upregulation of CnA occurs in response to AF-related tachycardia. Other studies have shown that increased atrial stretch may also upregulate Cn activity [46], highlighting the multi-factorial nature of AF-related Cn regulation. Both mRNA and protein levels of CnA β are significantly increased in cAF patients compared to sinus rhythm controls [44] and CnB expression is similarly enhanced [43].

3. Altered regulation of cardiac electrophysiology and contraction by protein phosphatases in AF

Figure 2 schematically depicts the protein phosphatase complexes regulating the local phosphorylation of individual ion channels and Ca²⁺-handling proteins, thereby modulating atrial electrophysiology and contraction in AF.

3.1. Modulation of APD by protein phosphatases

Reentry-promoting APD shortening is a hallmark of AF-associated atrial electrical remodeling and is mediated by transcriptional, translational and post-translational regulation of atrial ion channels [2]. A reduction in depolarizing I_{Ca,L}, likely developing as an adaptive response to the Ca²⁺-overload during initial stages of atrial tachycardia, is an important contributor to APD shortening in cAF patients [28] and ATR animal models [47]. Moreover, in the long term, this reduction likely contributes to the reduced Ca²⁺-transient amplitude and atrial hypo-contraction associated with AF [48–50]. The molecular mechanisms underlying reduced I_{Ca,L} are diverse [51], including, among other things, increased phosphatase-mediated channel dephosphorylation [28] (Figure 3). Similarly, indirect

evidence using pharmacological inhibitors identified impaired src-kinase regulation of $I_{Ca,L}$ together with an increased phosphatase activity, suggesting that a complex alteration in the kinase/phosphatase balance leads to $I_{Ca,L}$ dysregulation in cAF patients [29]. The PP2A-catalytic subunit can bind directly to the pore-forming L-type Ca^{2+} -channel α_{1C} -subunit, in close proximity to the PKA phosphorylation site Ser1928, but this interaction can be further modulated by multiple regulatory subunits and other phosphatases [52,53]. Disruption of PP2A binding using inhibitory peptides increase $I_{Ca,L}$, suggesting an important inhibitory role for channel-bound PP2A [53]. Similarly, data from guinea pig ventricular membrane patches suggest that PKA and phosphatases attached on or near the L-type Ca^{2+} -channel regulate basal $I_{Ca,L}$ [54]. However, inhibition of PP2A by okadaic acid increases open probability of single $I_{Ca,L}$ channels only in sinus rhythm patients, but not in cAF patients, despite the increase in global PP2A activity [27], whereas the same procedure increases whole-cell $I_{Ca,L}$ stronger in cAF than in sinus rhythm patients [28], suggesting that the mechanisms of PP2A-mediated $I_{Ca,L}$ regulation may be more complex and could differ in specific subpopulations of AF patients. Moreover, phosphorylation of the Ser1928 site does not appear to be required for β -AR-mediated regulation of $I_{Ca,L}$ and various other potential phosphorylation sites have been proposed [55]. Thus, the molecular mechanisms acutely regulating $I_{Ca,L}$ remain incompletely understood. Phosphatases may also reduce expression of L-type Ca^{2+} channels. In particular, Ca^{2+} -dependent activation of Cn and subsequent dephosphorylation of nuclear factor of activated T-cells (NFAT) promote translocation of NFAT to the nucleus, inhibiting transcription of L-type Ca^{2+} -channels [45,56]. This may contribute to the AF-related reduction in L-type Ca^{2+} -channel expression in some [27,57,58], but not all patient cohorts [28,29].

The total inward-rectifier K^+ current is larger in cAF patients due to both increased I_{K1} and the development of a receptor-independent, constitutive $I_{K,ACh}$ [59,60]. The increase in I_{K1} appears in large part due to upregulation of Kir2.1/Kir2.3 protein expression, which may be partly mediated by the Ca^{2+} /Cn/NFAT pathway. In particular, nuclear translocation of NFAT inhibits transcription of the inhibitory microRNA miR-26, thereby de-repressing Kir2.1 expression [61]. In addition, okadaic acid produced a small reduction in basal inward-rectifier K^+ -current in cAF, but not sinus rhythm patients [62], providing indirect evidence that the AF-related increase in phosphatases may also acutely augment the basal inward-rectifier K^+ -current. In contrast, PP2A (but not PP1 or Cn) is a component of the type-2 muscarinic-receptor complex that counteracts receptor phosphorylation (inactivation) by G-protein-coupled receptor kinases, thereby augmenting $I_{K,ACh}$ [63]. Accordingly, $I_{K,ACh}$ was smaller in the presence of okadaic acid in atrial cardiomyocytes from sinus rhythm patients [62], but open probability of constitutively active $I_{K,ACh}$ channels was increased [64]. In cAF, however, okadaic acid did not affect carbachol-activated $I_{K,ACh}$ suggesting potentially impaired function of G-protein-coupled receptor kinases [62] and highlighting the complexity of phosphorylation-dependent regulation of $I_{K,ACh}$ in cAF.

Augmentation of a number of other repolarizing currents may contribute to APD shortening in cAF. Increased I_{Ks} upon PKA-dependent phosphorylation contributes to AP-shortening during sympathetic stimulation and I_{Ks} is increased in cAF patients [65], with additional evidence for altered sympathetic regulation of I_{Ks} [66]. However, this increase has been attributed to enhanced β_1 -AR expression [66] and there is no information about altered

dephosphorylation of I_{Ks} by PP1, which is specifically targeted to the I_{Ks} macromolecular complex by the anchoring protein Yotiao [67], in cAF patients. We have recently identified I_{K2P} as an atrial-predominant K^+ -current involved in APD regulation [68]. Expression of $K_{2P3.1}$ channels and functional I_{K2P} were increased in cAF patients in our cohort, contributing significantly to AF-associated APD shortening [68]. By contrast, others have found reduced I_{K2P} in a cohort of AF patients with mitral valve disease, which could be rescued by exogenous application of phosphatases [69], suggesting that abnormal regulation of K_{2P} channel phosphorylation may affect I_{K2P} in certain patient populations. However, also for I_{K2P} it is unknown whether this abnormal phosphorylation is due to reduced dephosphorylation and, if so, which phosphatases and anchoring proteins are involved.

In contrast to I_{K1} , I_{Ks} and I_{K2P} , I_{to} is decreased in most cAF patients [66,70–73] and I_{Kur} in some [66,72,73], but not all studies [70,71], contributing to a prolongation of early atrial repolarization and resulting in the typical triangular shape of the atrial AP in cAF patients. CaMKII inhibition with KN-93 abolishes the differences in I_{to} between sinus rhythm and cAF patients [74], suggesting that the pore-forming subunit Kv4.3 of I_{to} might be CaMKII hyperphosphorylated in cAF patients. Whether reduced local phosphatase activity also contributes is unknown. Application of okadaic acid increased the sustained component of the outward K^+ -current (consisting for a large part of I_{Kur}) in atrial cardiomyocytes from sinus rhythm patients [74]. Like I_{CaL} , Cn is known to inhibit functional expression of I_{to} in cardiomyocytes [56], so the increased Cn activity in cAF may furthermore contribute to the reduced I_{to} .

The Na^+ - K^+ -ATPase is electrogenic, producing a repolarizing current that contributes to regulation of atrial APD and reentry dynamics [75]. PKA-dependent phosphorylation of Ser68 and PKC-dependent phosphorylation of Ser63 or Ser68 on phospholemman reduce its inhibition of the Na^+ - K^+ -ATPase [76]. PP1, but not PP2A, dephosphorylates phospholemman at Ser68 and this is controlled by the PP1 inhibitor I-1 [77]. Phospholemman phosphorylation at both Ser63 and Ser68 is decreased in rabbits with rapid atrial pacing (RAP)-induced atrial remodeling (Table 2), which would be expected to reduce Na^+ - K^+ -ATPase activity and contribute to intracellular Na^+ and Ca^{2+} overload [78], but the role of potential phosphatase remodeling was not addressed and data from human atrial samples are not available.

3.2. Modulation of cell-to-cell communication by protein phosphatases

Reduced I_{Na} , loss of electrical cell-to-cell communication via gap-junctions or interruption of muscle-bundle integrity by fibrosis all impair conduction and promote reentrant activity that can maintain AF [2]. Gap-junction proteins consist of connexins, which contain multiple phosphorylation sites for several kinases [79,80]. Both PP1 and PP2A co-localize with connexins and increased phosphatase activity in HF decreases gap-junction coupling through multiple mechanisms [81]. In a large animal model of AF, connexin gene transfer restores conduction velocity and prevents AF [82]. In cAF patients, the overall immunocytochemical staining of connexin 40 and connexin 43 in atrial samples is reduced, but lateral staining is significantly increased [83]. In agreement, connexins are dephosphorylated and redistributed to the lateral membranes in human atrial samples from

patients with atrial dilatation, a common feature in AF patients [84]. However, these findings are independent of the underlying rhythm [84]. In contrast, a different study observed increased connexin 40 phosphorylation, but reduced total connexin 40 levels, in AF patients with atrial dilatation compared to sinus rhythm patients with or without atrial dilatation [85], suggesting that other factors may further modulate connexin phosphorylation.

Atrial hypertrophy and fibrosis are major components of atrial structural remodeling that promote reentrant activity [1,2,10]. The AF-associated increase in Cn activity likely contributes to atrial hypertrophy via activation of the NFAT pathway [44,56]. Furthermore, this pathway may be involved in remodeling of the extracellular matrix in response to atrial stretch through activation of matrix metalloproteases [46]. In agreement, atrial samples from cAF patients are characterized by pronounced changes in collagen synthesis and degradation pathways, including increased expression of matrix metalloproteases [86].

Dephosphorylation of a set of conserved serine residues on class IIa histone deacetylases by a PP2A complex, consisting of C α , A α and B55 α subunits, controls their nuclear translocation where they repress the activity of prohypertrophic genes in the ventricle [87], but the precise roles of PP1/PP2A in atrial hypertrophy and fibrosis remain largely unknown.

3.3. AF-related modulation of Ca²⁺-handling by protein phosphatases

SR Ca²⁺ release occurs via RyR2 channels. In addition to post-translational regulation by oxidation and S-nitrosylation, the RyR2 channel is phosphorylated on Ser2030 and Ser2808 by PKA, and on Ser2814 by CaMKII [88]. Dephosphorylation of these sites is mediated by PP1, which is targeted to the complex by its regulatory subunit spinophilin-1 (PPP1R9B) [89], and by PP2A, targeted through its regulatory subunit PR130 (PPP2R3A) [67,90], through B56 α binding to ankyrin-B [91], or through the muscle-specific A-kinase-anchoring protein (mAKAP/AKAP6), which binds both RyR2 and PP2A-B56 δ (PPP2R5B) [92]. RyR2 phosphorylation at both Ser2808 and Ser2814 is increased in cAF patients [50,93–95], and Ser2814 phosphorylation (but not Ser2808 phosphorylation) is increased in most animal models with increased AF susceptibility (Table 2). Although the functional relevance of RyR2 hyperphosphorylation remains somewhat controversial, accumulating evidence suggests that RyR2 hyperphosphorylation promotes SR Ca²⁺ leak, spontaneous Ca²⁺-release events and DADs [88,96], thereby increasing the likelihood of triggered activity. Although the RyR2 hyperphosphorylation in cAF patients is likely in part due to increased PKA and CaMKII activity [50,95], a reduction in RyR2-associated phosphatases may also contribute. For example, microRNA-1 and microRNA-133-mediated reduction in B56 α expression results in reduced RyR2-associated PP2A levels in HF [90]. Furthermore, recent work has shown that mice with reduced PP1 in the RyR2 complex due to genetic knock-out of spinophilin-1 have increased RyR2 phosphorylation at Ser2814 (but unchanged Ser2808 phosphorylation), more spontaneous SR Ca²⁺-release events and an enhanced susceptibility to pacing-induced AF [89]. Although global spinophilin-1 mRNA levels are unchanged in cAF patients [39], our preliminary data suggest that the amount of PP1 associated to the RyR2 complex is decreased in cAF patients [97]. Finally, the Thr35-hyperphosphorylation of I-1 in cAF patients is expected to reduce PP1 activity in the SR microdomain, contributing to RyR2 hyperphosphorylation [12,37]. Taken together, the decreased local phosphatase activity in the RyR2 microdomain likely facilitates triggered-activity-promoting

Ca²⁺-handling abnormalities in cAF. By contrast, RyR2 phosphorylation at Ser2814 is not different in pAF patients compared to sinus rhythm controls, whereas Ser2808-mediated RyR2 phosphorylation is strongly reduced [31]. RyR2 open probability is nonetheless increased, potentially due to non-phosphorylation-related changes in the macromolecular complex [98]. Interestingly, dephosphorylation of RyR2 through exogenous application of phosphatases also increases SR Ca²⁺ leak, suggesting that increased phosphorylation and dephosphorylation of RyR2 both may enhance RyR2 [99]. Clearly much more molecular work is needed to dissect the precise role of altered RyR2 phosphorylation for channel function in health and disease.

SR Ca²⁺ uptake is decreased in cAF patients, potentially contributing to the smaller Ca²⁺-transient amplitude and atrial hypocontractility [50]. This reduced SR Ca²⁺ uptake is likely mediated in part by reduced SERCA2a expression and increased phospholamban (PLB) expression, resulting in a significant increase in PLB-mediated SERCA2a inhibition in cAF [39]. On the other hand, despite the increase in global PP1 and PP2A activities, phosphorylation of PLB at both Ser16 (PKA-site) and Thr17 (CaMKII-site) is higher in cAF patients, which should reduce the inhibitory consequences of enhanced total PLB (Table 2). Similarly, the expression of sarcolipin is reduced in cAF patients, which would increase SERCA2a activity [100]. The increased PLB phosphorylation is likely in part due to enhanced Thr35-phosphorylation (activity) of I-1, reducing PP1-mediated PLB dephosphorylation [39]. PP1 β is the most significant PP1 isoform involved in regulating SR Ca²⁺ cycling in rat cardiomyocytes [101], but other studies found that individual genetic deletion of PP1 α , PP1 β or PP1 γ does not alter PLB phosphorylation [102], suggesting partial redundancy of individual isoforms. The R_{GL} (PPP1R3A) subunit targets PP1 to the SR, where it controls dephosphorylation of PLB, but R_{GL} mRNA levels are unchanged in cAF patients [39]. Overexpression of B56 α results in reduced PLB phosphorylation during β -AR stimulation, suggesting a potential role for B56 α in targeting PP2A to PLB [103]. In contrast to cAF, SR Ca²⁺ uptake is increased in pAF, possibly due to increased Ser16-phosphorylation of PLB, contributing to increased SR Ca²⁺ load and the related increase in proarrhythmic spontaneous SR Ca²⁺-release events [31]. Furthermore, different AF animal models exhibit either decreased, unchanged or increased PLB phosphorylation (Table 2), making the interpretation of the role of altered phosphatase-mediated PLB dephosphorylation for AF paradigms challenging.

3.4. Modulation of contraction by protein phosphatases

In addition to the reduced Ca²⁺-transient amplitude, remodeling of contractile proteins, including altered expression of titin and myosin heavy chain isoforms, may contribute to AF-associated changes in atrial contractility [104,105] (Figure 3). Atrial contractility is also regulated by phosphorylation of contractile proteins, including the inhibitory troponin subunit (TnI), which influences Ca²⁺-sensitivity of the myofilaments and cMyBP-C, which determines Ca²⁺-sensitivity and kinetics of cross-bridge cycling [106]. cAF patients appear to have increased overall phosphorylation of troponin-T [104,105], cMyBP-C and desmin [105]. Increased phosphorylation of atrial light chain-2, the atrial-specific variant of myosin light chain-2, has been reported in some [105], but not all [104] studies.

PP1 and PP2A are the major phosphatases controlling dephosphorylation of myofilament proteins. PP1 preferentially dephosphorylates Ser23/24 on TnI, whereas PP2A induces a more uniform dephosphorylation [107]. Among the PP1 isoforms, PP1 β appears to be preferentially targeted to the myofilaments and loss of PP1 β increases phosphorylation of myosin light chain 2 and cMyBP-C [102]. The B56 α subunit may contribute to PP2A targeting to the myofilaments, with B56 α overexpression resulting in reduced TnI and cMyBP-C phosphorylation [103]. TnI phosphorylation at Ser23/24 is unchanged in cAF patients compared to sinus rhythm controls [105] and in multiple animal models of AF (Table 2). cMyBP-C phosphorylation at Ser282 is increased in cAF patients compared to sinus rhythm controls with dilated atria [105], but is decreased in a different cohort when compared to sinus rhythm patients with normal atrial dimensions [39] and in multiple AF animal models (Table 2). These data suggest complex remodeling of myofilament phosphorylation in response to AF and atrial dilatation, which are likely region specific (e.g., in left vs. right atrium) [39,104,105].

4. Challenges and future perspectives

4.1 Challenges in the dissection of the precise role of specific phosphatases in AF

There is emerging evidence that altered phosphorylation of proteins involved in atrial electrophysiology and contraction contributes to atrial arrhythmogenesis in cAF patients (Figure 3). One major obstacle in the field of phosphatase research is the fact that the proper study of phosphatase function requires preserved physiological target protein phosphorylation, which is not always possible for technical reasons. In addition, although pharmacological studies using putative inhibitors of different phosphatases have provided valuable evidence for the potential role of protein phosphatases for cardiac regulation, their limited specificity precludes definite conclusions [34]. For example, okadaic acid inhibits PP3-PP6 phosphatases with a similar efficacy as PP2A, suggesting that the PP2A-ascribed roles for cardiac function might be biased by potential inhibition of additional phosphatases. However, even with improved pharmacological tools, it will remain challenging to identify the precise role of phosphatases in the regulation of individual ion channels or Ca²⁺-handling proteins, given the redundancy between phosphatases in the regulation of individual sites and the numerous phosphorylation sites that influence function of a single protein. Finally, the large variability between different clinical forms of AF, as well as between AF patients and animal models, makes generic statements about the role of phosphatases in AF challenging. Instead, sub-groups should be analyzed to determine the roles of atrial rate, dilatation and other (clinical) factors in the AF-related regulation of phosphatases.

4.2 Targeting phosphatases as therapeutic opportunities for AF

Inhibition of tyrosine kinase by ibrutinib, employed in patients with chronic lymphocytic leukemia or mantle cell lymphoma has been associated with significantly increased risk of AF [108]. Although increased global PP1 and PP2A levels may also contribute to the formation of an AF-promoting substrate, it is likely that global phosphatase inhibition (e.g., using pharmacological inhibitors of catalytic subunits) will not be effective and may have pronounced adverse side effects, including promotion of AF itself. Direct and specific

targeting of holoenzymes (e.g., through the associated regulatory subunits), on the other hand, may allow regulation of the phosphorylation state of a specific substrate, and this is the most frequently pursued strategy for phosphatase drug discovery. Several examples of specific modulators for PP1-regulatory subunits for the treatment of diabetes [109], hypertension [110], Parkinson's disease or drug addiction [111] have been described. In addition, several studies have shown the therapeutic potential of protein phosphatase modulation via adenoviral-mediated gene therapy in both small animal [37] and large animal HF models [112]. Thus, inhibition of phosphatases may represent a novel opportunity to treat cardiovascular diseases, including AF, in the near future.

Given the central role for Ca^{2+} -handling abnormalities in animal models of AF and atrial samples from AF patients, targeting the phosphorylation of Ca^{2+} -handling proteins could be a promising antiarrhythmic strategy [2,113]. Although the functional relevance of RyR2 hyperphosphorylation remains a topic of debate [88], the proarrhythmic nature of RyR2 mutations and the antiarrhythmic efficacy of some drugs modulating RyR2 activity, suggest it could be an interesting target [99,113]. Furthermore, although our knowledge is far from complete, the RyR2 macromolecular complex is perhaps the best characterized, with multiple potential targets to modulate RyR2 function and some information about how to selectively modulate individual phosphorylation sites via alterations in phosphatase holoenzymes. Interestingly, the RyR2 hyperphosphorylation suggests that increased PP1, PP2A and Cn activities might be anti- instead of proarrhythmic. This indicates that we should increase local phosphatase activity as a therapeutic strategy.

4.3 Future perspectives

The molecular basis of increased phosphatase activity in AF is unknown. Since phosphatase activity is similarly increased in both ATR animal models and AF patients (Table 1), it is very likely that the upregulation is the consequence of AF itself, potentially to prevent unphysiological hyperphosphorylation of cellular proteins. Conversely, CHF dogs have atrial structural remodeling, which also promotes AF development, and increased PP1 activity, suggesting that besides the high atrial rate the underlying structural heart disease can also increase PP1 activity. The hemodynamic changes during HF lead to atrial dilatation and stretch and stretching of cultured neonatal rat atrial cardiomyocytes results in an upregulation of Cn [114]. Abnormal atrial metabolism may also play an important role in AF-promoting atrial remodeling [115] and specific mitochondria-associated phosphatases regulate mitochondrial function and signaling [116,117], suggesting a potential role for mitochondrial phosphatases in AF. Thus, besides potential contribution to AF progression and maintenance, the increase in phosphatases associated with AF-predisposing clinical conditions may also contribute to AF induction. The increase in PP1 activity under AF-promoting conditions in the absence of clear changes in the expression of catalytic subunits (Table 1), suggests functional regulation of phosphatase activity, for instance through phosphorylation or other forms of post-translational regulation. The exact (neurohumoral) factors and signaling pathways involved and their remodeling in AF will require further study. Finally, currently most information about phosphatase mediated regulation of atrial electrophysiology and contractility is based on global associations using pharmacological phosphatase inhibition, with limited information about the direct relationship between

phosphatase function and specific phosphorylation sites (perhaps with the exception of RyR2 Ser2808 and Ser2814). Recent work has identified a large number of putative PP1 regulatory subunits [30]. Future studies will likely help to obtain a better overview of the specific holoenzyme compositions responsible for targeting phosphatases to individual substrates. Taken together, further extensive work is required to dissect the precise role of PP1, PP2A and Cn in AF promotion and persistence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Atrial fibrillation (AF) is associated with profound atrial remodeling
- Global cardiomyocyte PP1, PP2A and calcineurin activities are increased in AF patients
- PP1, PP2A and calcineurin are involved in proarrhythmic atrial remodeling
- Methodological challenges exist to elucidate the precise roles of phosphatases in AF
- Phosphatases represent a potential therapeutic target for AF

Fig. 1. Schematic overview of the general concepts of AF pathophysiology

AF is induced and maintained by ectopic activity and reentry. Reentry requires an arrhythmogenic substrate and a suitable trigger to initiate it. The trigger usually involves premature beats or rapid ectopic firing. Numerous risk factors including genetics and cardiovascular and non-cardiovascular comorbidities cause alterations in atrial function and structure (remodeling) increasing the likelihood of ectopic activity and reentry. The remodeled substrate include effective refractory period (ERP) changes, conduction slowing and atrial fibrosis, ectopic activity is believed to result from abnormal automaticity, early (EADs) and delayed (DADs) afterdepolarizations and activation of autonomic nervous system (ANS). Increased activity of serine/threonine protein phosphatases, altered local phosphatase targeting and the subsequent alterations in protein phosphorylation are

suggested to mediated the effects of atrial remodeling promoting the induction and maintenance of AF by increasing the likelihood of ectopic activity and reentry.

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Fig. 2. Schematic overview of protein phosphatase complexes regulating the local phosphorylation of individual ion channels and Ca²⁺-handling proteins
Adapted from Heijman et al. [12].

Fig. 3. Schematic overview of altered protein phosphorylation and phosphatase activity in cAF patients

Table 1

Alterations in PP1 activity and expression in AF patients and animal models.

PP1				
		Human pAF	Human cAF	Experimental Models
Enzymatic activity			+62% [29] +49% [28] (PP1+PP2A) +48% [39]	Goat: ↔ [118] Dog ATR: +28% [119] Dog CHF: +83% [120]
Global protein levels		↔[30]	↔[27,28]	Goat: ↔ [118] Dog ATR: ↔ [119] Dog CHF: ↔ [120]
Specific subunits:				
PPP1CA (PP1 α)	mRNA		-37%[28]	
	Protein	↔[31]	+150%[39]	Rabbit RAP: +32% [121]
PPP1CB (PP1 β)	mRNA		-48%[28]	
PPP1CC (PP1 γ)	mRNA		↔[28]	
PPP1R1A (I-1)	mRNA		↔[39]	
	Phospho		Rel T35 +800%[39] Rel S67 ↔[39]	
PPP1R2 (I-2)	mRNA	↔[30]	↔[39]	
PPP1R3A (R _{GL})	mRNA		↔[39]	
PPP1R9B (spinophilin-1)	mRNA		↔[39]	
PP2A				
		Human pAF	Human cAF	Experimental Models
Enzymatic activity			+60%[29] +49%[28] (PP1+PP2A) +38%[39]	Goat: ↔[118] Dog ATR: ↔[119] Dog CHF: ↔[120]
Global protein levels		↔[31]	↔[27] +57%[28] +30%[39]	Goat: ↔[118] Dog ATR: ↔[119] Dog CHF: ↔[120]
Specific subunits:				
PPP2CA (PP2A-C α)	mRNA		-58%[28]	
	Protein			Rabbit RAP: ↔[121]
PPP2CB (PP2A-C β)	mRNA		↔[28]	
PPP2R3A (PR130)	mRNA		↔[39]	
PPP2R1A (PP2A-A/PR65)	Protein		↔[28]	
PP2B (Calcineurin)				
		Human pAF	Human cAF	Experimental Models
Enzymatic activity			+80%[44]	Goat: +272%[122] Dog ACMs (3Hz): +151%[45] Rat: Stretched ACMs: +76%[46] Human: In vitro paced atrial tissue (2Hz): +72%[44]
Global protein levels				Rat: Stretched ACMs: +43%[46]

PP1				
		Human pAF	Human cAF	Experimental Modelsn
Specific subunits:				
PPP3CA (CnA- α)	mRNA	+60%[43]	+68%[43] +55%[123]	
	Protein	+140%[43]	+215%[43] +37%[123] (60kD) +32%[123] (45kD)	Human: In vitro paced atrial tissue (2Hz): +39%[44];
PPP3CB (CnA- β)	mRNA		+55%[44] +60%[123]	Human: In vitro paced atrial tissue (2Hz): +100%[44]
	Protein		+83 [44]	
PPP3R (CnB)	mRNA	+236%[43]	+285%[43]	
	Protein	+164%[43]	+300%[43]	

ACM: atrial cardiomyocytes; ATR: atrial tachycardia remodeling; CHF: congestive heart failure; RAP: rapid atrial pacing

Table 2

AF-associated changes in phosphorylation

	Phospho Site	Human AF	Animal Model	Kinase	PP	Reference
Sarcoplasmic reticulum proteins (SR)						
RyR2	Ser2808	cAF: ↑; pAF: ↓	RAP: ↑ TG Mice: ↔ AVB: ↔ AF: ↔ CHF: ↔ AoB: ↔ ATR: ↔	PKA	PP1, PP2A	Greiser et al., JCI;2014 [78] Chiang et al., Cardiovasc Res;2014 [89] Vest et al., Circulation;2005 [93] Chelu et al., JCI;2009 [94] Voigt et al., Circulation;2012 [50] Neef et al., Circ Res;2010 [95] Li et al., Circulation;2014 [124] Guo et al., JCE;2014 [125] Shan et al., Circ Res;2012 [126] Li et al., Circ Res;2012 [127] Greiser et al., JMCC;2009 [118] Yeh et al., Circ Arrhythmia Electrophysiol;2008 [120] Zhang et al., PloS One;2015 [128] Lugenbiel et al., Plos One;2015 [129] Wakili et al., Circ Arrhythmia Electrophysiol;2010 [119]
	Ser2814	cAF: ↑; pAF: ↔	RAP: ↔, ↑ TG Mice: ↑ AF: ↑ AVB: ↑ CHF: ↔ AoB: ↔ ATR: ↔	CaMKII	PP1, PP2A	
	Ser2030			RAP: ↔	PKA	PP1, PP2A
PLB	Ser16	pAF: ↑	AF: ↓ AVB: ↓ RAP: ↔ CHF: ↔ AoB: ↔ ATR: ↔	PKA	PP2A	Voigt et al., Circulation; 2014 [31] Chelu et al., JCI;2009 [94] Neef et al., Circ Res;2010 [95] Li et al., Circulation;2014 [124] Greiser et al., JCI;2014 [78] Li et al., Circ Res;2012 [127] Greiser et al., JMCC;2009 [118] Yeh et al., Circ Arrhythmia Electrophysiol;2008 [120] El-Armouche et al., Circulation;2006 [39] Zhang et al., PloS One;2015 [128] Lugenbiel et al., Plos One;2015 [129] Wakili et al., Circ Arrhythmia Electrophysiol;2010 [119]
	Thr17	cAF: ↑; pAF: ↔	RAP: ↔ TG Mice: ↔, ↑ AF: ↔ AVB: ↔ CHF: ↑ ATR: ↔	CaMKII	PP1, PP2A	
Plasma membrane proteins						
PLM	Ser63		RAP: ↓	PKA	PP1	Greiser et al., JCI;2014 [78]
	Ser68		RAP: ↓	PKC	PP1	
Myofilament proteins						
TnI	Ser23/24	cAF: ↔	RAP: ↓ CHF: ↔ ATR: ↔ AF: ↔ AVB: ↓	PKA, PKC	PP1, PP2A	Yeh et al., Circ Arrhythmia Electrophysiol;2008 [120] El-Armouche et al., Circulation;2006 [39] Belus et al., Circ Res; 2010 [105] Greiser et al., JMCC;2009[118] Greiser et al., JCI;2014 [78]

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	Phospho Site	Human AF	Animal Model	Kinase	PP	Reference
						Wakili et al., Circ Arrhythmia Electrophysiol;2010 [119]
cMyBPC	Ser282	cAF: ↓, ↑	CHF: ↓ ATR: ↓ AF: ↔ AVB: ↓ RAP: ↔	PKA, PKC	PP1, PP2A	Yeh et al., Circ Arrhythmia Electrophysiol;2008 [120] El-Armouche et al., Circulation;2006 [39] Belus et al., Circ Res;2010 [105] Greiser et al., JMCC;2009 [118] Greiser et al., JCI;2014 [78] Wakili et al., Circ Arrhythmia Electrophysiol;2010 [119]

ATR: atrial tachycardia remodeling; AVB: atrio-ventricular block; CaMKII: Ca²⁺/calmodulin-dependent protein kinase-II; CHF: congestive heart failure; PKA: protein kinase-A; PKC: protein kinase-C; RAP: rapid atrial pacing; TG: transgenic;