Drug Targets

IN-DEPTH FOCUS

2 GPCRs: new opportunities and challenges for drug discovery
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8 Ion channels: novel therapeutic targets
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G protein-coupled receptors (GPCRs) are by far the most diverse and therapeutically relevant class of receptors in the human genome. It is estimated that around 30% of all currently marketed drugs target GPCRs. Emerging concepts in GPCR pharmacology such as ‘allosteric modulation’ and ‘functional selectivity’ have led to significant efforts in identifying and optimising receptor ligands with novel mechanisms of action. In this review, we will focus on the concept of ‘allosterism’ and the implications for the discovery, validation and development of a new generation of GPCR-targeted therapeutics.

GPCRs generate responses to multiple ligands by binding and activating intracellular heterotrimeric G proteins that subsequently interact with intracellular effector systems such as adenylate cyclase or phospholipase C, leading to a wide variety of distinct physiological responses. To protect cells against overstimulation, the vast majority of GPCRs ‘switch off’ upon prolonged exposure to agonists through the process of ‘desensitisation’, whereby the response of a given receptor declines over time despite the presence of agonists. Most drugs targeting GPCRs act at the binding site of the natural or endogenous ligand (orthosteric site), and therefore compete with the endogenous ligand to either stimulate (agonist / partial agonist) or inhibit (antagonist / inverse agonist) receptor activity. Antagonists have no activity on their own (neutral antagonists) but can compete with agonists to inhibit receptor signalling, whereas inverse agonists have the ability to behave as antagonists, but can also inhibit receptor ‘constitutive activity’ (basal activity in the absence of agonist). In the ‘conformational model’ of GPCRs, receptors exist in equilibrium between an inactive and active conformational state in which a continuum of active states exist, ranging from having no activity to being maximally active. Simplicistically, at one end of the spectrum, full agonists bind with high affinity and stabilise the
active state, and at the other end, inverse agonists favour and stabilise the inactive conformation.

Typically, ligand efficacy is defined by a ligand’s capacity to activate a single signalling pathway. However, it is now well established that any given ligand for a GPCR does not simply possess a single defined efficacy; rather, a ligand possesses multiple efficacies depending on the specific downstream signal transduction pathway being investigated. This diversity is believed to be the result of conformational changes induced in the GPCR that are ligand-specific and this phenomenon is referred to as ‘functional selectivity’ or ‘ligand-bias’\(^3,4\). Hence, receptors can adopt various conformations that preferentially activate / modulate one signalling pathway to the exclusion of others.

Allosteric modulators, ligands that bind to sites topographically distinct from the orthosteric site (Figure 1A, page 4) are believed to stabilise or induce changes in the receptor active state causing a shift in responsiveness to their endogenous ligands\(^5\). Allosteric modulation of GPCRs by endogenous substrates is well documented; for example Na\(^+\), Ca\(^{2+}\) and Zn\(^{2+}\) ions have been shown to modulate ligand binding and functional properties of a wide range of GPCRs\(^5\). Similarly, L-amino acids, glycine and amidated lipids have also been found to modulate certain GPCRs in an allosteric manner\(^7\). It should also be noted that GPCRs themselves are allosteric by nature in that they interact with their cognate G proteins, accessory proteins, ion channels and other GPCRs (homo or heterodimerisation), demonstrating that GPCRs possess multiple sites for allosteric interactions\(^8\).

Small molecule allosteric modulators have been identified for numerous GPCRs and are classified as follows: positive allosteric modulators (PAMs) enhance the affinity and/or efficacy of the receptors natural ligand whereas negative allosteric modulators (NAMs) attenuate the effects of the natural ligand (Figure 1B, page 4). Classically, allosteric modulators have been defined as relying on the presence of the endogenous ligand to exert their effects, however, some allosteric modulators have been shown to exhibit intrinsic activity and are referred to as ‘ago-allosteric’ modulators (Figure 1B, page 4). A fourth class of allosteric ligand has been described that binds to the receptor but has no apparent effect on endogenous ligand activity and is referred to as a ‘silent allosteric modulator’ (SAM)\(^10,11\). Importantly, since orthosteric and allosteric ligands bind to topographically distinct sites of the receptor, both ligands can interact with the receptor simultaneously and thus, each ligand can affect the binding (binding cooperativity) and the intrinsic activity (activation cooperativity) of the other. Theoretical models describing these interactions have been described extensively elsewhere\(^12-14\).

Unique properties of allosteric modulators

Allosteric modulators have a unique range of properties compared to orthosteric ligands and thus can offer multiple advantages over classic orthosteric agonists or antagonists. Firstly, the effects of allosteric modulators (PAMs or NAMs) are saturable, meaning they can be administered at relatively high concentrations without over-stimulating or over-inhibiting the system, as when the receptor is fully occupied by an orthosteric ligand, the allosteric ligand, even in
excess, can have no further effect. This is often referred to as the ‘ceiling’ effect. From a clinical perspective, the saturability effect would greatly reduce the risk of overdose. Secondly, allosteric modulators, as they rely on the presence of the endogenous ligand, have the ability to modify receptor activity in a spatial and temporal manner. PAMs in particular would be anticipated to exert their effects only when and where the endogenous ligand is present. Their ligand binding domains because they have evolved to accommodate a common natural ligand. Conversely, allosteric binding sites within receptor subtypes tend to be less well conserved as they are theoretically under less evolutionary pressure. This characteristic of allosteric binding sites within a receptor species can facilitate the identification of subtype-selective modulators, and is exemplified by the muscarinic receptors for which ‘absolute subtype selectivity’ has been achieved. Conventional orthosteric agonists of the muscarinic receptors have shown clinical utility in the treatment of various CNS disorders, however, the lack of subtype-selectivity and associated side-effects has prevented clinical development of these drugs. Pioneering the way for muscarinic receptor subtype-selectivity, Lazarenko et al discovered that thiochrome, a metabolite of thiamine, binds allosterically to all five muscarinic receptors but only enhances the affinity of acetylcholine for M4 receptors, having no effect on acetylcholine affinity at any of the other muscarinic receptor subtypes. Furthermore, the selective action on M4 was corroborated in GTPγS functional binding assays, clearly demonstrating that subtype-selectivity at the muscarinic receptors is obtainable. The mAChRs together with mGluRs are probably the most well studied GPCR families with respect to allosteric modulation, for which a full range of allosteric ligands (PAMs, NAMs and ago-allosteric modulators) have been identified and have the potential to be clinically useful in multiple therapeutic indications such as pain, depression and anxiety, epilepsy, Parkinson’s disease, drug abuse, and schizophrenia. Thus, selectively targeting a receptor subtype while sparing related receptors involved in other physiological processes can enable the development of drugs with improved safety profiles. Although the lack of evolutionary conservation of allosteric sites provides an excellent mechanism of action is thus in synchrony with the frequency and magnitude of physiological signaling, which is in stark contrast to the continuous and global activation of the targeted receptor that can occur with an orthosteric agonist. This suggests that allosteric modulators are less likely to induce side-effects and furthermore, because activation of the receptor is not constant, they have less propensity to promote receptor desensitisation, so are less likely to induce drug tolerance; a therapeutic liability. Recent studies have shown that the GABAA receptor (GABAAR) positive allosteric modulators CGP7930 and GS39783 for example are devoid of the sedative, hypothermic and muscle relaxant effects of the orthosteric agonist baclofen.

Thirdly, allosteric modulators can provide subtype selectivity. Subtypes of a particular receptor species such as the five muscarinic acetylcholine receptors (mACHR1-5) or the eight metabotropic glutamate receptors (mGluR1-8) typically share high amino acid sequence homology in their respective endogenous

**Figure 1** Schematic representation of GPCR allosteric modulation. A. Allosteric modulators (AM) bind to GPCRs at sites that are topographically distinct from the orthosteric ligand (OL) binding site and stabilise a ‘new’ receptor conformation leading to changes in affinity (α) and/or efficacy (β) of the orthosteric ligand. B. Allosteric ligands can modulate orthosteric agonist affinity (α) as indicated by a shift in agonist CRCs, to the left (PAM) or to the right (NAM). In addition, allosteric ligands also modulate orthosteric agonist efficacy (β) as indicated by an upward or downward shift of agonist maximal efficacy. Finally, some allosteric modulators also exhibit ago-allosteric activity as indicated by an increase in signal in the absence of orthosteric ligand.
is that the allosteric interaction can vary depending on the nature of the orthosteric ligand used to probe receptor activity, a behaviour known as ‘probe dependence’

In many physiological systems, this behaviour should not pose a problem, assuming that there is only a single endogenous ligand for the receptor of interest. However, there are numerous examples in which individual GPCRs can respond to multiple endogenous ligands, and these include the melanocortin receptors and glucagon-like 1 receptor (GLP1-R). Recently, Koole et al investigated two GLP1-R PAMs (Novo Nordisk ‘compound 2’ and the natural flavonoid, quercetin) for their ability to modify binding and signalling of multiple endogenous GLP1-R ligands, and the GLP1 mimic Exendin-4. Interestingly, ‘compound 2’ was found to selectively enhance cAMP signalling and did so in a probe-dependent manner having the greatest effect on oxyntomodulin, whereas quercetin selectively modulated calcium signalling had effects only on truncated GLP1 peptides or Exendin-4 and no effects on oxyntomodulin or full-length GLP1. Thus, compound 2 and quercetin exhibit not only probe-dependence but also pathway-dependence. In addition to probe- and pathway-dependence, the behaviour of an allosteric modulator can also be influenced by the system used to evaluate its effects; this is referred to as ‘context-dependence’. As mentioned earlier, GPCR activity is also modulated by the receptor’s interaction with cognate G proteins, and accessory proteins etc.

So the pharmacology of the allosteric ligand will also be influenced by receptor expression levels and the complement of regulatory proteins available in the system used. Therefore, the behaviour of the allosteric modulator may be very different in a recombinant cell line versus the native expression system.

Allosteric modulation of GPCR activity is a complex phenomenon, as allosteric ligands can alter the biological properties of the endogenous ligand by modulating the affinity and/or efficacy and may even possess intrinsic activity. This becomes even more complex when there are multiple endogenous ligands to consider (as is the case for the GLP-1R) because the allosteric interaction can vary with the nature of the orthosteric ligand (probe dependence). Further complicating the issue is that these allosteric effects can also be pathway- and system-dependent (Figure 2, page 6). Ideally, the endogenous or a physiologically relevant probe and cell system should be used wherever possible when characterising the effects of allosteric modulators on receptor activity in order for experimental data to be predictive in vivo.

**Identifying allosteric modulators**

Traditionally, high-throughput screening (HTS) assays used to identify small molecule GPCR modulators relied on competition assays that detect the ability of the test compound to displace a radiolabelled endogenous ligand. This strategy is biased towards the discovery of agonists / antagonists acting at the orthosteric site where a ‘hit’ would be identified if there were an alteration in the binding of a radiolabelled orthosteric ligand in the presence of the test compound. As allosteric interactions with the receptor may not necessarily affect orthosteric site occupancy, such allosteric modulators would go undetected, hence the paucity of allosteric modulators in the clinic. In the mid-late 1990s, radioligand binding HTS assays were superseded with cell-based functional assays that monitor receptor-G protein interactions and subsequent
downstream signalling events such as changes in intracellular CAMP or Ca^{2+} levels. The advantage of these functional assays is that they have the potential to detect a wider spectrum of ligand efficacies, as the only prerequisite is that the compound perturbs receptor function irrespective of where it binds. The same assay formats can be successfully employed for the identification of allosteric modulators^{11,35,36}. For example, GPCR cell-based functional assays designed to detect PAMs are modified to include submaximal concentrations of endogenous agonists that result in about 20 per cent of maximal response (EC_{20}). This allows the propensity of the co-administered synthetic ligand to potentiate or change the quality of the endogenous signal to be determined. Following confirmation of hits identified in this screening paradigm, compounds displaying an effect on the endogenous ligand EC_{20} response are evaluated for their effects in full concentration response curves (CRC) of endogenous ligand. A compound exhibiting PAM activity would be expected to shift the CRC of the endogenous ligand to the left (increase potency) and/or upwards (enhance efficacy) as illustrated in Figure 1B.

However, a major drawback to this approach is that conventional cell-based functional HTS assays only detect the signal defined by the assay system (CAMP, or Ca^{2+}, etc.). This becomes an issue when considering functional selectivity, whereby various ligands of the same receptor can stabilise distinct receptor conformations and preferentially activate/silence downstream pathways. Therefore, when developing a functional assay to detect small molecules targeting GPCRs, 'context' or system-based signalling should be considered.

References

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The follicle stimulating hormone (FSH) receptor

**Clinically relevant GPCR allosteric modulators**

To date, allosteric modulators have been identified for at least 35 GPCR types and subtypes, some targeting previously intractable receptors, GLP1-R for example. Allosteric modulation of GPCRs promises to have a favourable impact on future drug discovery efforts, and has already delivered two marketed drugs with the potential to yield many more. The first GPCR allosteric ligand to appear in the clinic was Cinacalcet (Sensipar™), a positive allosteric modulator (PAM) of the CaSR. Cinacalcet, by interacting with the transmembrane regions of the CaSR (distal to the orthosteric binding domains in the N-terminus) enhances affinity for Ca2+ regulating altered parathyroid hormone (PTH) levels associated with hyperparathyroidism and hypocalcinemia. More recently, Maraviroc (Celsentry™) obtained FDA approval for the treatment of HIV. Maraviroc is an allosteric inhibitor of chemokine receptor 5 (CCR5) that stabilises a CCR5 conformation unable to bind HIV-1, and thereby inhibits HIV-1 entry into host cells. Importantly, maraviroc has only minor effects on CCR5 affinity for CCL3L1, an endogenous ligand of CCR5.

**Concluding remarks**

This broad overview of GPCR allosteric modulation covers the concept of GPCR allosterism and the implications it has on the drug discovery process. Allosteric modulation of GPCRs offers tremendous benefits with respect to both efficacy and safety of future therapies targeting this receptor family, including the potential for greater receptor subtype selectivity and the ability to ‘fine-tune’ physiological responses. Identifying such modulators can be relatively straightforward, however the development of such drugs requires characterisation of the specific mechanisms being modulated and as such, GPCR allosteric modulators may also pose significant challenges beyond those seen with orthosteric modulators. Despite these challenges, the exploitation of GPCR allosteric binding sites as alternative drug targets has become more commonplace in current drug discovery efforts. Moreover, Cinacalcet and Maraviroc have demonstrated ‘proof-of-principle’ in humans and confirmed that allosteric modulators are clinically relevant molecules and as such have opened the floodgates for the development of a new generation of GPCR-targeted therapeutics.

**Biographies**

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Chloride channels and cardiac arrhythmia: novel therapeutic targets?

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The equilibrium potential for Cl\(^{-}\) is within a membrane potential range (usually \(-65\) to \(-40\) mV) that is more positive than the resting membrane potential (RMP) and can be either negative or positive to the actual membrane potential during the normal cardiac cycle. Thus, compared with cationic (Na\(^{+}\), K\(^{+}\), Ca\(^{2+}\); channels, Cl\(^{-}\) channels have the unique ability to generate both inward and outward currents through the same channel and cause both depolarisation and repolarisation during the action potential. Therefore, activation of Cl\(^{-}\) channels may cause significant abbreviation of APD and depolarisation of RMP that can induce early afterdepolarisation and cause arrhythmias under pathological conditions. However, no specific pharmacological agents are currently available for manipulating Cl\(^{-}\) channel functions in the heart. In this review, we summarise the current understanding of the emerging role of Cl\(^{-}\) channels in cardiac electrophysiology and arrhythmogenesis. We also describe advances in translational research that may identify Cl\(^{-}\) channels as novel therapeutic targets for the treatment of cardiac arrhythmias.

To date, at least eight different types of Cl\(^{-}\) currents have been discovered in the myocytes from different regions of the heart and in different species\(^1\). At the molecular level, all cardiac Cl\(^{-}\) channels described so far may fall into the following four categories\(^1\) (Figure 1, page 10): 1) the cystic fibrosis transmembrane
conductance regulator (CFTR), which is a member of the adenosine triphosphate-binding cassette (ABC) transporter superfamily and may be responsible for the Cl- currents activated by cAMP ($I_{Cl,cAMP}$), protein kinase A (PKA) ($I_{Cl,PKA}$), protein kinase C (PKC) ($I_{Cl,PKC}$), and extracellular ATP ($I_{Cl,ATP}$); 2) CLC-2, which is a member of the CLC voltage-gated Cl- channel superfamily and may be responsible for the hyperpolarisation- and cell swelling-activated inwardly rectifying Cl- current ($I_{Cl,ir}$); 3) CLC-3, which is also a member of the CLC Cl- channel superfamily and may be responsible, directly or indirectly, for the volume-regulated outwardly rectifying Cl- current ($I_{Cl,vol}$), including the basally activated ($I_{Cl,b}$) and swelling activated ($I_{Cl,swell}$) components, and also a Cl-/H+ antiporter; and 4) the Ca2+-activated Cl- channels (CACCs), the candidate genes include the TMEM16A (anoctamin 1 or Ano1), CLCA-1, and Bestrophin, which are responsible for the Ca2+-activated Cl- current ($I_{Cl,Ca}$).

Under physiological conditions, the intracellular Cl- concentration ($[Cl^-]_i$) in cardiac myocytes is between 10 to 20 mmol/L as estimated by analysis of intracellular Cl- activity ($a_{Cl}^i$) using ion-selective microelectrode2-5. With an extracellular Cl- concentration ($[Cl^-]_o$) of 145 mmol/L, therefore, the equilibrium potential for Cl- ($E_{Cl}$) is within a membrane potential range (usually –65 to –40 mV) that is more positive than the resting membrane potential and can be either negative or positive to the actual membrane potential during the normal cardiac cycle. Thus, compared with cationic (K+, Na+, Ca2+ etc.) channels, cardiac Cl- channels have the unique ability to generate both inward and outward currents through the same channel and cause both depolarisation and repolarisation during the action potential. Therefore, activation of Cl- channels may produce significant effects on cardiac pacemaker activity and action potential characteristics (Figure 2, page 10).

The degree to which activation of Cl- currents depolarises the resting membrane or accelerates the repolarisation of action potential depends critically on the actual value of $E_{Cl}$ and the magnitude of the Cl- conductance relative to the total membrane conductance. Under physiological conditions, for example, the activation of CLC-3 and CFTR Cl- channels in the heart will result in outwardly rectifying currents because the transmembrane Cl- gradient is asymmetrical. This will have more significant effects at positive potentials to accelerate repolarisation and cause a shortening of the APD compared with smaller depolarising effects at negative potentials near the RMP (Figure 2, page 10). Thus, activation of CLC-3 or CFTR channels will result in a shortening of Q-T interval (Figure 2, page 10). The ability of Cl- current activation to depolarise cardiac cells is also opposed by the presence of a large background K+ conductance that normally controls the resting membrane potential. Both abbreviation of APD and depolarisation of $E_m$ upon activation of Cl- channels may induce early afterdepolarisation (EAD) and play a role in arrhythmogenesis under pathological conditions (Figure 2, page 10).

CFTR channels and arrhythmias

CFTR channels are closed under basal conditions and are activated only when the intracellular...
PKA- and PKC-dependent phosphorylation activity is increased, such as under conditions of β-adrenergic, purinergic, or histamine stimulation. Telemetry ECG recordings revealed no significant difference in ECG parameters between CFTR+/− mice and their wild-type littermates, which is consistent with the low basal activity of CFTR channels in the heart. Recent studies showed cardioprotective effect of activation of CFTR channels against ischemia / reperfusion-induced myocardial injury. CFTR may play a crucial role in ischemic preconditioning and postconditioning protection of the heart from myocardial infarction. It is not clear, however, whether activation of CFTR channels also produces electrophysiological benefit and reduces cardiac arrhythmias during ischemia / reperfusion. With asymmetric CI gradients, under physiological conditions, cardiac CFTR channels carry an outwardly-rectifying current, with a smaller depolarising inward current and a larger repolarising outward current (Figure 2). Therefore, a major physiological role of activation of CFTR may be to prevent excessive APD prolongation and protect the heart against the development of EAD and triggered activity caused by activation of Ca2+ channels during β-adrenergic stimulation. EADs arising from phase 2 and 3 underlie focal triggered tachyarrhythmias and repolarisation abnormalities, which contribute to cardiac sudden death. It is well-established that APD prolongation favours EADs by allowing recovery of inward currents and, conversely, shortening of APD makes it more difficult to induce EADs. It has been shown that activation of CFTR channels by endogenous catecholamine release contributes to hypoxia-induced shortening in APD. Therefore, activation of CFTR channels should protect against focal triggered arrhythmias.

However, under some pathologic conditions, activation of CFTR may cause arrhythmias. For example, when background K+ conductance is reduced in the case of myocardial hypokalaemia, activation of CFTR may cause depolarisation and induce abnormal automaticity. Activation of CFTR channels will cause significant membrane depolarisation and induce abnormal automaticity. Activation of CFTR channels may accelerate the development of re-entry due to...
CLC-2 channels and arrhythmias

CLC-2 channels are activated by hyperpolarisation, cell swelling and acidosis and have an inwardly rectifying $I-V$ relationship (Figure 1, page 10). During the cardiac action potential, therefore, the CLC-2 channel will conduct mainly an inward current as a result of Cl$^-$ efflux at negative membrane potentials and cause a depolarisation of the RMP of cardiac cells. At membrane potentials more positive than $E_D$, CLC-2 channels may conduct a small outward current as a result of Cl$^-$ influx and may accelerate repolarisation of the action potential. It is also possible that, in a manner analogous to the role and tissue distribution pattern of the cationic pacemaker channels ($I_h$), the hyperpolarisation-activated inwardly rectifying Cl$^-$ current ($I_{Cl,ir}$) through CLC-2 channels may play a much more prominent role in the sinoatrial (SA) or atrioventricular (AV) nodal regions of the heart (Figure 3). It has been consistently shown that ischemia and acidosis depolarise the RMP of cardiac myocytes, increase automaticity and cause lethal arrhythmias, although the mechanism has remained obscure. CLC-2 channels are sensitive to extracellular acidosis and cell volume and activation of CLC-2 channels by ischemia- or hypoxia-induced acidosis and cell swelling could be responsible for these phenomena and be pro-arrhythmic. Therefore, it may be possible that the significance of $I_{Cl,ir}$ in the heart may be of pathological importance during hypoxia- or ischemia-induced acidosis or cell swelling. Drugs targeting CLC-2 channels could be anti-arrhythmic. In conscious CLC-2 knockout (CLCn2$^{-/-}$) mice, telemetry ECG analysis revealed an increased incidence of AV block and a decreased chronotropic response to acute exercise stress when compared to their age-matched wild-type (CLCn2$^{+/+}$) and heterozygous (CLCn2$^{+-}$) littermates (Figure 3). Targeted inactivation of CLC-2 does not alter intrinsic heart rate but prevents the positive chronotropic effect of acute exercise stress through a sympathetic regulation of CLC-2 channels$^{11}$.

CLC-3 channels and arrhythmias

The Cl$^-$ current through the volume-regulated Cl$^-$ channels (VRCCs) under basal or isotonic conditions is small but can be further activated by stretching of the cell membrane by inflation or direct mechanical stretch of membrane $\beta_1$-integrin and/or cell swelling induced by exposure to hypoosmotic solutions$^{12,13}$. Activation of VRCCs is expected to produce depolarisation of the RMP and significant shortening of APD because of its strong

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"Telemetry ECG recordings revealed no significant difference in ECG parameters between CFTR$^-$ mice and their wild-type littermates, which is consistent with the low basal activity of CFTR channels in the heart"
outwardly rectifying property (Figure 1 on page 10 and Figure 4). Because cardiac myocytes swell during hypoxia and ischemia, and the washout of hyperosmotic extracellular fluid after reperfusion induces further cell swelling, activation of VRCCs may contribute to APD shortening and arrhythmias induced by hypoxia, ischemia and reperfusion. Shortening of APD, therefore, the effective refractory period (ERP) reduces the cycle length of the conducting pathway needed to sustain re-entry (wavelength) (Figure 2, page 10). In principle, this favours the development of atrial fibrillation (AF) or ventricular fibrillation (VF), depending on the presence of multiple re-entrant circuits or rotating spiral waves. Activation of $I_{\text{vol}}$ may slow or enhance the conduction of early extrasystoles, depending on the timing. Perfusion with the VRCC blocker indanyloxyacetic acid-94 reversed organised VF to complex VF with lower frequencies, indicating that VRCC underlies the changes in VF dynamics. Consistent with this interpretation, CLC-3 channel protein expression is 27% per cent greater on left than right ventricles, and computer simulations showed that insertion of $I_{\text{vol}}$ transformed complex VF to a stable spiral. Therefore, activation of $I_{\text{vol}}$ has a major impact on VF dynamics by transforming random multiple wavelets to a highly organised VF with a single dominant frequency. In the case of also inhibits $I_{\text{Ca}}$, was almost equivalent to the effect of tamoxifen on APD and EAD in these myocytes. It has been shown that mechanical stretching or dilation of the atrial myocardium is able to cause arrhythmias. Since $I_{\text{Ca}}$ was also found in 5-A nodal cells, VRCCs may serve as a mediator of mechanotransduction and play a significant role in the pacemaker function if they act as the stretch-activated channels in these cells. Therefore, the consequences of activation of $I_{\text{vol}}$ are very complex. It may be detrimental, beneficial, or both simultaneous in different parts of the heart under different pathophysiological conditions (Figure 4).

**CaCCs and arrhythmias**

Even though $I_{\text{Ca}}$ is also expected to be outwardly rectifying under physiological conditions, the activation of $I_{\text{Ca}}$ will have considerably different effects on cardiac action potentials and RMP (Figure 5, page 13) from those of CFTR and CLC-3 channels (Figure 2, page 10). This is because the kinetic behaviour of $I_{\text{Ca}}$ is significantly determined by the time course of the $[\text{Ca}^{2+}]_{i}$ transient. Normally, $I_{\text{Ca}}$ will have insignificant effects on the diastolic membrane potential, as resting $[\text{Ca}^{2+}]_{i}$ is low. When $[\text{Ca}^{2+}]_{i}$ is substantially increased above the physiological resting level, however, activation of CaCCs generates a significant amount of transient outward current (Figure 1, page 10). $I_{\text{Ca}}$ will activate early during the action potential in response to an increase in $[\text{Ca}^{2+}]_{i}$, associated with $\text{Ca}^{2+}$-induced $\text{Ca}^{2+}$ release (ICR). The time course of decline of the $[\text{Ca}^{2+}]_{i}$ transient will determine the extent to which $I_{\text{Ca}}$ contributes to early repolarisation during phase 1. In the rabbit left ventricle, $I_{\text{Ca}}$ contributes to APD shortening in sub-endocardial myocytes but not in sub-epicardial myocytes. These differences in functional expression of $I_{\text{Ca}}$ may reduce the electrical heterogeneity in the left ventricle. In $\text{Ca}^{2+}$-overloaded cardiac preparations, $I_{\text{Ca}}$ may contribute to the arrhythmogenic transient inward current ($I_{\text{h}}$). $I_{\text{h}}$ produces delayed afterdepolarisation (DAD) and induces triggered activity, which is an important mechanism for abnormal impulse formation. In sheep Purkinje and ventricular myocytes, activation of $I_{\text{Ca}}$ was found to induce DAD and plateau transient repolarisation. Therefore, blockade of $I_{\text{Ca}}$ may be potentially anti-arrhythmogenic by reducing DAD amplitude and triggered activity based on DAD. However, the role of $I_{\text{Ca}}$ in phase 1 repolarisation and the generation of EAD

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**Figure 4 CLC-3 Cl channels in cardiac myocytes**

CLC-3, a member of voltage-gated CLC Cl channel family, encodes Cl channels in vascular smooth muscle cells that are volume regulated ($I_{\text{vol}}$) and can be activated by cell swelling ($I_{\text{swell}}$) induced by exposure to hypotonic extracellular solutions or possibly membrane stretch. $I_{\text{vol}}$ is a basally activated CLC-3 Cl current; α-helices of CLC-3 are shown as a red box. CLC-3 proteins are expressed on both sarcolemmal membrane and intracellular organelles including mitochondria (mito) and endosomes. The proposed model of endosome ion flux and function of Nox1 and CLC-3 in the signalling endosome is adapted from Miller Jr et al. Binding of IL-1 β or TNF-α to the cell membrane initiates endocytosis and formation of an early endosome (EEA1 and Rab5), which also contains NADPH oxidase subunits Nox1 and p22phox, in addition to CLC-3. Nox1 is electrogenic, moving electrons from intracellular NADPH through a redox chain within the enzyme into the endosome to reduce oxygen to superoxide. CLC-3 functions as a chloride–proton exchanger, required for charge neutralisation of the electron flow generated by Nox1. The ROS generated by Nox1 result in NF-κB activation. Both CLC-3 and Nox1 are necessary for generation of endosomal ROS and subsequent NF-κB activation by IL-1 β or TNF-α in VSMCs. Statins block CLC-3 channels, which causes hyperpolarisation of the cell membrane, closure of $\text{Ca}^{2+}$ channels and vasorelaxation, and inhibition of cell proliferation. PKC, protein kinase C; PT, serine–threonine protein phosphatases; α-AR, α-adrenergic receptor; Gi, heterodimeric inhibitory G protein. Nox: NADPH oxidase.

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**“Under pathophysiological conditions, the contribution of Cl channels to the regulation of cardiac action potential may be greater and may be an important mechanism for the ionic remodelling”**

Myocardial hypertrophy and heart failure, ionic remodelling is one of the major features of pathophysiological changes. Under these conditions, $I_{\text{vol}}$ is constitutively active. The persistent activation of $I_{\text{vol}}$ may limit the APD prolongation and make it more difficult to elicit EAD. Indeed, in myocytes from failing hearts, blocking $I_{\text{vol}}$ by tamoxifen significantly prolonged APD and decreased the depolarising current required to elicit EAD by about 50 per cent. And hyper-osmotic cell shrinkage, which
Range of zero-current values corresponding to mechanism for the ionic remodelling. No support that Cl- channels may contribute be determined.

Changes in action potentials (top) and membrane (CaCCs) in heart

Figure 5 Modulation of cardiac electrical activity by activation of Ca2+-activated Cl channels (CaCCs) in heart

In summary, emerging data strongly support that CaCC channels may contribute significantly to the cardiac electrophysiology through its unique properties of generating both inward and outward currents during action potential. Under pathophysiological conditions, the contribution of CaCC channels to the regulation of cardiac action potential may be greater and may be an important mechanism for the ionic remodelling. No doubt, CaCC channels in the heart may be novel therapeutic targets for the treatment of cardiac arrhythmias as well as other cardiovascular disease. However, there is a long way before clinical application and the following important issues need to be resolved.

(1) The drugs (blockers or agonists) with higher pharmacological-specificity on individual cardiac Cl channels need to be developed.

The lack of specific pharmacological tools to effectively separate the individual Cl channels not only has hampered the understanding of Cl channel function in cardiac physiology and pathophysiology but also bring up other unpredictable side effects on other transport proteins and signalling pathway. At the same time, the drugs used in experimental trials, such as tamoxifen, one drug for the prevention and treatment of breast cancer, and doxy-cycline, a kind of antibiotics, might not be appropriate either as anti-arrhythmic drugs or as a medicine taken for a long period because of their side effects.

(2) The interactions with other cationic channels and among different Cl channels and compensatory changes during cardiovascular diseases need to be further clearly delineated. There are several types of Cl channels expressed concomitantly in the same cardiac cell. It is unclear how these Cl channels interact and whether there exists a compensatory mechanism among the multiple Cl channels to maintain intracellular Cl concentration and regulate APD.

(3) The other cellular functions of Cl channels and their effects after blockade or activation of Cl channels need to be clarified. For example, CLC-3 has versatile functions. It can strengthen the regulatory volume decrease (RVD) and protect cardiac myocytes from excessive increase in cell volume during hypoxia, ischemia or hypertrophy. It also prevents apoptosis by regulation of cell volume homeostasis. However, it is yet not known whether stimulation of CLC-3 activity would lead to electrophysiological benefit.

(4) The interaction of Cl channels with other Cl transporters or exchangers under patho-logic conditions need to be further elucidated. A variety of sarcocemmal anion co-transporters and exchange proteins are expressed in cardiac cells, which include Cl/HCO3− exchanger, Na+-dependent Cl transporter, K+-Cl co-transporter and Cl/OH exchanger. All of them take part in the balance of intracellular Cl homeostasis.

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References