

Approaching ryanodine receptor therapeutics from the calcin angle

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Release of Ca^{2+} from intracellular stores is a ubiquitous mechanism that contributes to numerous physiological processes, including secretion, transcription, apoptosis, and contraction. In many cases, the rise in intracellular Ca^{2+} is mediated by Ca^{2+} release channels called ryanodine receptors (RyRs) and involves a positive feedback mechanism known as Ca^{2+} -induced Ca^{2+} release (CICR). Just as normal RyR function is essential for life, abnormal RyR function can cause disease; notable examples include malignant hyperthermia (MH), central core disease, catecholaminergic polymorphic ventricular tachycardia, heart failure, and Alzheimer's disease (Betzenhauser and Marks, 2010; Chakroborty and Stutzmann, 2014; Ríos et al., 2015). Thus, development of new therapeutic RyR-targeting drugs carries substantial clinical potential. In this issue, Xiao et al. comprehensively characterize a relatively new family of RyR ligands, the calcins.

Several compounds have been examined as possible precursors of future RyR-targeting agents, the most famous being ryanodine, caffeine, and dantrolene. The ryanodine and caffeine actions are dramatic; consequently, these agents (to our knowledge) have not been extensively explored as potential precursors of future RyR therapies. Dantrolene and its derivatives are antagonists of RyR-mediated Ca^{2+} release (Ikemoto et al., 2001), and dantrolene itself is an FDA-approved drug for the treatment of MH and spasms in skeletal muscle. Interestingly, dantrolene also appears to have neuroprotective (Liang and Wei, 2015) and antiarrhythmic actions (Penttinen et al., 2015). At issue, however, is the uncertainty surrounding the mechanism by which dantrolene limits RyR-mediated Ca^{2+} release, which adds difficulty to assessing its potential as a precursor of future RyR-targeted agents. Another interesting class of RyR ligands is the benzothiazepine derivatives (e.g., JTV519 and S107). These agents purportedly stabilize the RyR-FK506 binding protein complex, but the underlying molecular mechanism remains controversial. This mechanistic uncertainty adds difficulty to predicting the potential of these agents as future therapeutic tools. On a positive note, the benzothiazepine derivatives do limit diastolic SR Ca^{2+} leak and thus could help normalize the abnormally high Ca^{2+} leak associated with heart failure (Betzenhauser and Marks, 2010).

More recently, the β -blocker carvedilol and its derivatives have been forwarded as possible precursors of future RyR-targeting agents (Zhou et al., 2011; Zhang et al., 2015). Individual RyR openings become shorter when these compounds bind to the RyR, which limits diastolic SR Ca^{2+} leak as well as the inter-RyR CICR that underlies dangerous propagating diastolic Ca^{2+} waves. The danger arises because Ca^{2+} waves can drive electrogenic Na^+ - Ca^{2+} exchange at the surface membrane that is sufficient to trigger delayed afterdepolarizations (DADs)—potentially life-threatening arrhythmogenic events. This antiwave action of carvedilol is unique among β -blockers but is unfortunately dose limited by its β -blocking capacity (Zhou et al., 2011). Therefore, carvedilol derivatives that shorten RyR openings without causing β -block have been generated (Zhou et al., 2011), and these have significant potential as precursors for the development of future RyR-targeting agents. Interestingly, The FDA-approved carvedilol (trade name: Coreg) is a racemic mixture that includes the non- β -blocking R-enantiomer, which shortens RyR openings. In addition, RyR up-regulation has been proposed to contribute to pathology in Alzheimer's disease mouse models (Chakroborty and Stutzmann, 2014), and carvedilol appears to slow cognitive deterioration associated with Alzheimer's disease (Wang et al., 2011), implying that non- β -blocking carvedilol derivatives may have additional clinical applications beyond the heart.

The RyR-targeting drugs described above all work to restrain RyR function. In contrast, the recent study by Xiao et al. (2016) characterizes and compares eight calcins that enhance RyR activity, which makes calcins an intriguing prospect for future RyR drug development.

The calcins

Calcins are an expanding group of small basic peptides (~4 kD) found in the venoms of certain scorpions. Imperatoxin was the first calcin to be identified (Valdivia et al., 1992) and was renamed imperacalcin after other calcins were found. Calcins share high primary sequence similarity ($\geq 78\%$) and are stabilized in a compact globular thermostable structure by an inhibitor cysteine knot (ICK) motif. A key feature of calcins is the large dipole

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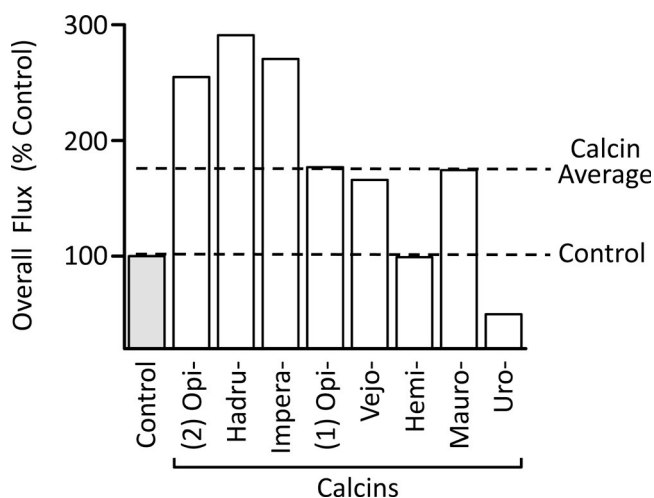


Figure 1. Calcin-modified RyR currents. Time-averaged flux carried by open control and calcin-modified RyR channels was estimated from the all points histograms presented in Xiao et al. (2016). These flux determinations are the sum of the full and subconductance currents (open probability considered) and have been normalized to the control value. The average of these eight calcin-modified current determinations is 185% that of the drug-free control RyR.

moment resulting from their highly segregated charge distribution. Despite this, calcins manage to penetrate the cell membrane (Gurrola et al., 2010) to reach their target, the RyR. Calcins bind to RyRs with high affinity (K_d values of 0.3–376 nM) and specificity (they have no other known targets). Interestingly, some calcins have a higher affinity for the RyR than ryanodine itself. Unlike ryanodine, however, the calcin–RyR dissociation rate is sufficiently fast to permit observation of calcin reversibility in single RyR studies. Xiao et al. (2016) report that calcins specifically activate skeletal RyR channels (RyR1s) via inducing long-lived subconductance states. They show that the fractional current amplitude of the subconductance state varies between ~20 and ~60% of full conductance, depending on which calcin is bound. Furthermore, they find that the subconductance state attributes bear no relationship to each calcin's dipole moment, RyR1 binding affinity, or effect on RyR1 Ca^{2+} sensitivity. Interestingly, calcins induce an additional subconductance state in RyRs during the ryanodine-modified subconductance state (Tripathy et al., 1998)—a sub-substate. This additive action is reasonable evidence that calcins and ryanodine bind to different sites on the RyR, which is not surprising considering the distinct chemical nature of these two ligands (peptide vs. alkaloid). As cell-permeating peptides, calcins could be used as vehicles to carry therapeutic cargo into cells (Boisseau et al., 2006). As high-affinity RyR ligands, calcins are also interesting precursors for the development of RyR-targeting drugs. Combined, these features make calcins incredibly interesting agents.

The action of calcins on RyR-mediated Ca^{2+} release has also been examined in cells, in particular, during diastole in ventricular cardiac muscle cells. In cardiomyocytes, single RyRs spontaneously open (albeit infrequently) to mediate SR Ca^{2+} leak. The frequency of these spontaneous diastolic openings increases as SR Ca^{2+} load increases. Because the steady-state diastolic SR Ca^{2+} load is ultimately set by the balance between SR Ca^{2+} leak and uptake, SR Ca^{2+} leak, uptake, and load are intimately interrelated. Ca^{2+} overload in the SR results in abnormally frequent or long spontaneous diastolic RyR openings and/or local bouts of inter-RyR CICR (sparks), which may evoke life-threatening propagating intracellular Ca^{2+} waves. Imperacalcin induces trains of sparks at discrete SR Ca^{2+} release sites in cardiac myocytes (Terentyev et al., 2002). This very interesting action is remarkably similar to the action of ryanodol in cardiac myocytes (Ramos-Franco et al., 2010), and like ryanodol, imperacalcin induces intermittent sojourns to a long-lived subconductance state in RyRs. It is thought that these agents likely introduce a sustained low-intensity Ca^{2+} release event at affected release sites, mediated by one (or a few) long-lived RyR subconductance opening or openings, repeatedly triggering sparks at that affected release site (Terentyev et al., 2002; Ramos-Franco et al., 2010). Assuming SR Ca^{2+} uptake rate remains constant, the increased diastolic SR Ca^{2+} leak will work to reduce SR Ca^{2+} load. This is consistent with the observed action of hadrucalcin in electrically paced cardiomyocytes (Schwartz et al., 2009), which reduces steady-state intracellular Ca^{2+} transient amplitudes, suggesting a lower SR Ca^{2+} load. Thus, it is reasonable to believe that calcins (or their descendent agents) could be used to increase SR Ca^{2+} leak and thus limit SR Ca^{2+} overload, a potentially deadly arrhythmogenic risk factor. Although the study of Xiao et al. (2016) nicely quantifies various calcin attributes, we would like to draw attention to some areas of calcin action that warrant additional discussion, which we hope will be thought provoking.

Calcins: Agonists or antagonists

Xiao et al. (2016) refer to calcins as RyR agonists even though calcin binding reduces the unitary current. Agents that reduce the current carried by an ion channel are traditionally called antagonists. However, the counter-intuitive interpretation of Xiao et al. (2016) does make sense because calcins increase the steady-state SR Ca^{2+} leak in cardiomyocytes (Terentyev et al., 2002). This clearly indicates that more Ca^{2+} , not less, is being released during diastole when calcins are present. Apparently, the long duration of the subconductance state makes up for the reduction in fractional current. Fig. 1 illustrates this graphically. We used the single RyR all points histograms presented in Xiao et al. (2016) to estimate the overall ion flux carried by control and cal-

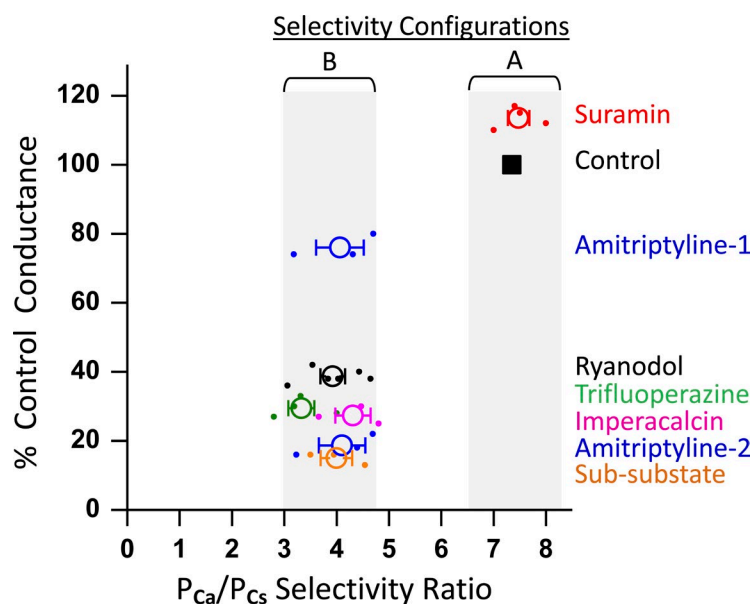


Figure 2. RyR selectivity configurations. Unitary RyR2 currents were measured with 25 mM Ca^{2+} on the luminal side of the channel and 100 mM Cs^{+} on the cytosolic side. Tested drugs were applied to the cytosolic solution. Subconductance states induced by ryanodol, imperatoxin, amitriptyline, and trifluoperazine were examined as was the superconductance state induced by suramin and the normal control full-open state in the absence of drug. The relative permeability of Ca^{2+} and Cs^{+} in these states was calculated using the measured current reversal potential and equation labeled no. 1 in Fatt and Ginsborg (1958). Individual RyR determinations are shown as small filled circles and the mean (\pm SE) selectivity as larger open circles. Method: Heavy SR microsomes were isolated from rat ventricle muscle as described elsewhere (Copello et al., 1997). Planar bilayers contained a 5:3:2 lipid mixture (50 mg/ml in decane) of phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine, respectively. Bilayers were formed across an \sim 100- μ m hole in a Teflon partition separating solution compartments (luminal and cytosolic). All chemicals and drugs were obtained from commercial sources. Ryanodol was generated from high purity commercially available ryanodine. Single channel currents were digitized at 20 kHz and filtered at 1 kHz using a Bessel filter for analysis. Voltage ramps were applied and current reversal potential determined.

cin-modified RyRs. On average, calcins increased the overall ion flux carried by the RyRs. Of course, how this translates into overall Ca^{2+} flux will depend on the relative permeation characteristics of the full open and calcin-induced subconductance states. Note that Xiao et al. (2016) applied the calcins at 100 nM, which is a near-saturating concentration for all except urocalcin, whose K_d is 376 nM.

Calcin-induced subconductance: A steric hindrance or allosteric mechanism

Xiao et al. (2016) call the intermittent long-lived RyR subconductance states the calcins' "signature effect." However, ryanodol has a very similar action on single RyR channels (Ramos-Franco et al., 2010), suggesting the action of calcins is not unique and that calcins and ryanodol may share a mechanistic commonality.

Xiao et al. (2016) acknowledge that the subconductance effect could be explained in various ways. Their preferred explanation, however, is that calcins enter the permeation pathway and bind to negative residues therein to either partially occlude (via steric hindrance) or rearrange the permeation pathway in a way that limits ion passage. The reason Xiao et al. (2016) favor this explanation is because imperacalcin binding varies with membrane potential (Tripathy et al., 1998), suggesting it may enter the electric field to reach its binding site. A second reason is that calcins are \sim 2.5–3.0 nm in diameter and could thus physically fit into the \sim 5-nm-wide cytosolic vestibule that leads to the RyR's central pore. Another reason is the near unitary Hill coefficient of the calcins' signature effect, which suggests there may

be a single calcin-binding site per channel, instead of multiple. The central pore could be that singular site. Interestingly, ryanodol's action on the RyR channel also has a Hill coefficient near one (Ramos-Franco et al., 2010), and mutagenesis experiments suggest the ryanoids interact with the RyR's central pore structure (Chen et al., 2002).

There are reasons, however, to interrogate the "enter the permeation pathway" hypothesis forwarded by Xiao et al. (2016). First, an early cryo-electron microscopy three-dimensional RyR1 reconstruction reported that imperacalcin may bind to an external site on the RyR located some distance (\sim 11 nm) from the central pore (Samsó et al., 1999). To physically visualize the bound calcin, however, it was linked to biotin/streptavidin/colloidal gold. This rather large tag could have prevented imperacalcin from entering into the permeation pathway or could have added substantial error to its given location (11 nm from the central pore). Second, chemically diverse agents (calcins and ryanodol) have strikingly similar actions (Tripathy et al., 1998; Schwartz et al., 2009; Ramos-Franco et al., 2010) on single RyRs and on diastolic RyR-mediated Ca^{2+} release in cardiomyocytes (Terentyev et al., 2002; Ramos-Franco et al., 2010). As suggested earlier, this implies some commonality of mechanism, which is difficult to envision given the disparity in size of these chemically diverse agents. Third, an early study by Lindsay et al. (1994) forwarded an allosteric explanation for the long-lived subconductance state induced by ryanodine. Specifically, they proposed that ryanodine binds outside the permeation pathway but still alters its conformation, and conse-

quently its function. Forth, Xiao et al. (2016) report that calcins induce subconductance states with different fractional current amplitudes, and these amplitudes have no apparent correlation to each calcin's dipole moment. This very interesting observation would be difficult to explain if calcins induced the subconductance state via a steric hindrance mechanism.

Various pharmacological agents are known to alter RyR conductance. Calcins (Tripathy et al., 1998; Schwartz et al., 2009) and ryanodol (Ramos-Franco et al., 2010) induce intermittent long-lived subconductance states. Amitriptyline (Zima et al., 2008) and trifluoperazine (Qin et al., 2009) induce short-lived subconductance states. Suramin induces a superconductance state (Sitsapesan and Williams, 1996; Liu, 2008). Over the years, we have applied these agents to single RyRs in planar lipid bilayers (Zima et al., 2008; Qin et al., 2009; Ramos-Franco et al., 2010), and an accumulated outcome arising from our experiments (Liu, 2008) is presented in Fig. 2. Here, the conductance of the normal RyR2 open state (square) as well as that of various drug-modified RyR2 open states (circles) are plotted as a function of ion selectivity (i.e., P_{Ca}/P_{Cs} ratio). One might expect that conductance would be inversely proportional to selectivity because, intuitively, the processes providing the high selectivity would most likely slow down ion translocation. This was not the case. Instead, we found that RyR2 appeared to assume one of two Ca^{2+} selectivity configurations (Fig. 2, A and B). RyR2's normal open state and the suramin superconductance state were associated with the higher Ca^{2+} selectivity configuration (Fig. 2, A). Conversely, RyR2's subconductance open states were all associated with the lower Ca^{2+} selectivity configuration (Fig. 2, B). The point labeled "sub-sub-state" represents the additive actions of imperacalcin and ryanodol. The resulting sub-substate has smaller current amplitude than the subconductance states induced when imperacalcin or ryanodol are added alone. However, the Ca^{2+} selectivity of the resulting sub-sub-state was like that of all the other subconductance states. In other words, the lower Ca^{2+} selectivity configuration (Fig. 2, B) appears to be a mechanistic commonality of all the subconductance states tested that is independent of the state's current amplitude or the agent that induced the state. Again, this is difficult to reconcile with a steric hindrance explanation for the subconductance states. We suggest the various agents may bind at different sites (maybe some inside the permeation pathway, others outside), but all allosterically alter the conformation of the RyR pore, which has two preferred conformations (as shown in Fig. 2).

Regardless of where the calcins bind (i.e., inside or outside the permeation pathway), the calcins are fascinating membrane-permeable high-affinity RyR agonists, and this makes them unique and interesting precursors for the development of future RyR-targeting drugs.

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REFERENCES

- Betzenhauser, M.J., and A.R. Marks. 2010. Ryanodine receptor channelopathies. *Pflugers Arch.* 460:467–480. <http://dx.doi.org/10.1007/s00424-010-0794-4>
- Boisseau, S., K. Mabrouk, N. Ram, N. Garmy, V. Collin, A. Tadmouri, M. Mikati, J.-M. Sabatier, M. Ronjat, J. Fantini, and M. De Waard. 2006. Cell penetration properties of maurocalcine, a natural venom peptide active on the intracellular ryanodine receptor. *Biochim. Biophys. Acta.* 1758:308–319. <http://dx.doi.org/10.1016/j.bbame.2006.02.007>
- Chakraborty, S., and G.E. Stutzmann. 2014. Calcium channelopathies and Alzheimer's disease: insight into therapeutic success and failures. *Eur. J. Pharmacol.* 739:83–95. <http://dx.doi.org/10.1016/j.ejphar.2013.11.012>
- Chen, S.R.W., P. Li, M. Zhao, X. Li, and L. Zhang. 2002. Role of the proposed pore-forming segment of the Ca^{2+} release channel (ryanodine receptor) in ryanodine interaction. *Biophys. J.* 82:2436–2447. [http://dx.doi.org/10.1016/S0006-3495\(02\)75587-2](http://dx.doi.org/10.1016/S0006-3495(02)75587-2)
- Copello, J.A., S. Barg, H. Onoue, and S. Fleischer. 1997. Heterogeneity of Ca^{2+} gating of skeletal muscle and cardiac ryanodine receptors. *Biophys. J.* 73:141–156. [http://dx.doi.org/10.1016/S0006-3495\(97\)78055-X](http://dx.doi.org/10.1016/S0006-3495(97)78055-X)
- Fatt, P., and B.L. Ginsborg. 1958. The ionic requirements for the production of action potentials in crustacean muscle fibres. *J. Physiol.* 142:516–543. <http://dx.doi.org/10.1113/jphysiol.1958.sp006034>
- Gurrola, G.B., E.M. Capes, F.Z. Zamudio, L.D. Possani, and H.H. Valdivia. 2010. Imperatoxin A, a cell-penetrating peptide from scorpion venom, as a probe of Ca -release channels/ryanodine receptors. *Pharmaceuticals (Basel)*. 3:1093–1107. <http://dx.doi.org/10.3390/ph3041093>
- Ikemoto, T., T. Hosoya, H. Aoyama, Y. Kihara, M. Suzuki, and M. Endo. 2001. Effects of dantrolene and its derivatives on Ca^{2+} release from the sarcoplasmic reticulum of mouse skeletal muscle fibres. *Br. J. Pharmacol.* 134:729–736. <http://dx.doi.org/10.1038/sj.bjp.0704307>
- Liang, L., and H. Wei. 2015. Dantrolene, a treatment for Alzheimer disease? *Alzheimer Dis. Assoc. Disord.* 29:1–5. <http://dx.doi.org/10.1097/WAD.0000000000000076>
- Lindsay, A.R., A. Tinker, and A.J. Williams. 1994. How does ryanodine modify ion handling in the sheep cardiac sarcoplasmic reticulum Ca^{2+} -release channel? *J. Gen. Physiol.* 104:425–447. <http://dx.doi.org/10.1085/jgp.104.3.425>
- Liu, Y. 2008. Ryanodine receptor function in intracellular calcium signaling. PhD dissertation. ProQuest/UMI #3345086. Loyola University Chicago, Chicago. 126 pp.
- Penttinen, K., H. Swan, S. Vanninen, J. Paavola, A.M. Lahtinen, K. Kontula, and K. Aalto-Setälä. 2015. Antiarrhythmic Effects of dantrolene in patients with catecholaminergic polymorphic ventricular tachycardia and replication of the responses using iPSC models. *PLoS One*. 10:e0125366. (published erratum appears in *PLoS One*. 2015. <http://dx.doi.org/10.1371/journal.pone.0134746>) <http://dx.doi.org/10.1371/journal.pone.0125366>

- Qin, J., A.V. Zima, M. Porta, L.A. Blatter, and M. Fill. 2009. Trifluoperazine: a ryanodine receptor agonist. *Pflugers Arch.* 458:643–651. <http://dx.doi.org/10.1007/s00424-009-0658-y>
- Ramos-Franco, J., A.M. Gomez, A. Nani, Y. Liu, J.A. Copello, and M. Fill. 2010. Ryanodol action on calcium sparks in ventricular myocytes. *Pflugers Arch.* 460:767–776. <http://dx.doi.org/10.1007/s00424-010-0839-8>
- Ríos, E., L. Figueroa, C. Manno, N. Kraeva, and S. Riazzi. 2015. The couplonopathies: A comparative approach to a class of diseases of skeletal and cardiac muscle. *J. Gen. Physiol.* 145:459–474. <http://dx.doi.org/10.1085/jgp.201411321>
- Samsó, M., R. Trujillo, G.B. Gurrola, H.H. Valdivia, and T. Wagenknecht. 1999. Three-dimensional location of the imperatoxin A binding site on the ryanodine receptor. *J. Cell Biol.* 146:493–500. <http://dx.doi.org/10.1083/jcb.146.2.493>
- Schwartz, E.F., E.M. Capes, E. Diego-García, F.Z. Zamudio, O. Fuentes, L.D. Possani, and H.H. Valdivia. 2009. Characterization of hadrucalcin, a peptide from *Hadrurus gertschi* scorpion venom with pharmacological activity on ryanodine receptors. *Br. J. Pharmacol.* 157:392–403. <http://dx.doi.org/10.1111/j.1476-5381.2009.00147.x>
- Sitsapasan, R., and A.J. Williams. 1996. Modification of the conductance and gating properties of ryanodine receptors by suramin. *J. Membr. Biol.* 153:93–103. <http://dx.doi.org/10.1007/s002329900113>
- Terentyev, D., S. Viatchenko-Karpinski, H.H. Valdivia, A.L. Escobar, and S. Györke. 2002. Luminal Ca^{2+} controls termination and refractory behavior of Ca^{2+} -induced Ca^{2+} release in cardiac myocytes. *Circ. Res.* 91:414–420. <http://dx.doi.org/10.1161/01.RES.0000032490.04207.BD>
- Tripathy, A., W. Resch, L. Xu, H.H. Valdivia, and G. Meissner. 1998. Imperatoxin A induces subconductance states in Ca^{2+} release channels (ryanodine receptors) of cardiac and skeletal muscle. *J. Gen. Physiol.* 111:679–690. <http://dx.doi.org/10.1085/jgp.111.5.679>
- Valdivia, H.H., M.S. Kirby, W.J. Lederer, and R. Coronado. 1992. Scorpion toxins targeted against the sarcoplasmic reticulum Ca^{2+} -release channel of skeletal and cardiac muscle. *Proc. Natl. Acad. Sci. USA.* 89:12185–12189. <http://dx.doi.org/10.1073/pnas.89.24.12185>
- Wang, J., K. Ono, D.L. Dickstein, I. Arrieta-Cruz, W. Zhao, X. Qian, A. Lamparello, R. Subnani, M. Ferruzzi, C. Pavlides, et al. 2011. Carvedilol as a potential novel agent for the treatment of Alzheimer's disease. *Neurobiol. Aging.* 32:2321.e1–2321.e12. <http://dx.doi.org/10.1016/j.neurobiolaging.2010.05.004>
- Xiao, L., G.B. Gurrola, J. Zhang, C.R. Valdivia, M. SanMartin, F.Z. Zamudio, L. Zhang, L.D. Possani, and H.H. Valdivia. 2016. Structure–function relationships of peptides forming the calxin family of ryanodine receptor ligands. *J. Gen. Physiol.* <http://dx.doi.org/10.1085/jgp.201511499>
- Zhang, J., Q. Zhou, C.D. Smith, H. Chen, Z. Tan, B. Chen, A. Nani, G. Wu, L.-S. Song, M. Fill, et al. 2015. Non- β -blocking R-carvedilol enantiomer suppresses Ca^{2+} waves and stress-induced ventricular tachyarrhythmia without lowering heart rate or blood pressure. *Biochem. J.* 470:233–242. <http://dx.doi.org/10.1042/BJ20150548>
- Zhou, Q., J. Xiao, D. Jiang, R. Wang, K. Vembaiyan, A. Wang, C.D. Smith, C. Xie, W. Chen, J. Zhang, et al. 2011. Carvedilol and its new analogs suppress arrhythmogenic store overload–induced Ca^{2+} release. *Nat. Med.* 17:1003–1009. <http://dx.doi.org/10.1038/nm.2406>
- Zima, A.V., J. Qin, M. Fill, and L.A. Blatter. 2008. Tricyclic antidepressant amitriptyline alters sarcoplasmic reticulum calcium handling in ventricular myocytes. *Am. J. Physiol. Heart Circ. Physiol.* 295:H2008–H2016. <http://dx.doi.org/10.1152/ajpheart.00523.2008>