buffering of the Ca²⁺ load. There are two main classes of Ca²⁺-permeable channel that mediate Ca²⁺ influx, in addition to some other more minor Ca²⁺ entry pathways.

There is a family of voltage gated Ca²⁺ channels (VGCC) that is highly selective for Ca²⁺ under normal circumstances.² It is well established that plasma membrane depolarization results in a very rapid rise in [Ca²⁺], mediated by these channels.³⁻⁴ Characteristically, these channels also inactivate rapidly, too. Although the inactivation characteristics vary considerably between members of this family of channels, even the longest opening channels (such as L-type, or high-voltage activated [HVA] channels) inactivate with a half time of a few seconds.⁵ Pharmacological manipulation of VGCC in many cases is readily accomplished, and is also of therapeutic significance. L-type channels have a well established pharmacological profile, and can be blocked by dihydropyridines (such as nifedipine and nimodipine), phenylalkylamines (verapamil) and benzothiazepines (diltiazem), all of which are in clinical use for the treatment of hypertension and angina.⁶,⁷ Several other clinically used agents, including the antidiarrhoal loperamide, also possess L-type channel blocking activity.⁸ Although their laboratory use is sometimes complicated by voltage-and use-dependent mechanisms of inhibition,⁹ L-type channels are very accessible to pharmacological modification. Attacking the other classes of VGCC presents more of a problem. The cone snail-derived toxin ω-conotoxin GVIA is an irreversible inhibitor of N-type channels, while ω-conotoxin MVIC has a broader spectrum of activity and may block N, P and Q-type channels.¹⁰,¹¹ The more limited availability of agents to block this more recently described group of VGCC subtypes can make it more difficult to define the participation of a specific channel subtype in a given signaling pathway. Interestingly, T-type channels are the target of ethosuximide, and may be the basis of the anti-epileptic activity of this drug.¹²

In addition to voltage-gated channels there is also a large class of ligand gated channels that facilitate Ca²⁺ entry. These include several sub-types of glutamate and nicotinic receptors amongst others.¹³⁻¹⁵,¹⁶,¹⁷ These channels are typically less selective for Ca²⁺ compared to VGCC, and their activation often results in a substantial rise in intracellular free Na⁺, too.¹⁸ This can impact on the effectiveness of efflux pathways, and will be discussed below. Interestingly, the relative permeability of these channels to Ca²⁺ over Na⁺ is governed by the subunit composition, so that altering the molecular make-up of a channel can have a substantial impact on the Ca²⁺ permeability.¹⁵,¹⁶ It is widely recognized that activation of glutamate receptors, and in particular N-methyl-D-aspartate (NMDA) receptors as well as Ca²⁺ permeable non-NMDA receptors, can result in the accumulation of

**Introduction**

Many intracellular signaling events are triggered by transient changes in the cytoplasmic free Ca²⁺ concentration ([Ca²⁺]). Ca²⁺-triggered events are critical for both normal cellular activity and for pathophysiologica changes in cell function. It might be reasonable to assume, then, that the manipulation of the processes that govern the passage of Ca²⁺ through the cytoplasm could have a substantial impact on cellular activity. There are many pharmacological tools available to perform experiments that would allow this assumption to be tested. However, making an accurate prediction about the impact of modifying Ca²⁺ buffering requires an understanding of the various mechanisms involved in the regulation of [Ca²⁺], and also an appreciation of the benefits and limitations of the pharmacological tools that are available. This review will focus on Ca²⁺ modulating processes with a focus on neurons. From an organizational standpoint it is convenient to divide the text into Ca²⁺ influx, Ca²⁺ release and Ca²⁺ efflux pathways. It is important to remember, however, that these distinctions are somewhat arbitrary given that many of the mechanisms are either bi-directional, or are tightly coupled to compensatory pathways that may link Ca²⁺ influx or depletion to Ca²⁺ entry.

**Ca²⁺ Entry Pathways**

Most cells are endowed with a variety of voltage- and ligand-gated ionic channels that normally serve as the principle Ca²⁺ entry pathway for cells. Activation of these ion channels allows the entry of Ca²⁺ down a substantial electrochemical gradient, with the result that the cytoplasmic free Ca²⁺ concentration is elevated above the normal resting range of 50 - 100 nM.³ The magnitude of the elevation depends on the characteristics of activation and inactivation of the channel, the ionic selectivity of the channel, and the overall balance between the entry and the subsequent
pathological amounts of Ca\(^{2+}\) in neurons. The effectiveness of glutamate receptors in this regard comes from the incomplete inactivation of NMDA receptors, so that the reasonably high Ca\(^{2+}\) permeability is coupled with prolonged periods of Ca\(^{2+}\) entry. The resulting very large Ca\(^{2+}\) load is neurotoxic.\(^{19, 20}\) Interestingly, Ca\(^{2+}\)-mediated neurotoxicity can also be accomplished pharmacologically by preventing the desensitization of non-NMDA receptors, which also resist a large Ca\(^{2+}\) load.\(^{21}\) The pharmacological properties of this family of Ca\(^{2+}\)-permeable ligand gated ion channels are beyond the scope of this review, but it is appropriate to anticipate that the magnitude of [Ca\(^{2+}\)] changes should correspond to the level of activation of the receptor of interest, and that inhibition of the receptor should correspondingly prevent increases in [Ca\(^{2+}\)].

**Ca\(^{2+}\) Release Mechanisms**

An important mechanism for elevating [Ca\(^{2+}\)] that is at least partially independent from Ca\(^{2+}\) entry involves the release of Ca\(^{2+}\) from intracellular stores. Most cells are endowed with endo- or sarcosplasmic reticular specializations that allow the accumulation, storage and release of Ca\(^{2+}\).\(^{22}\) The accumulation of Ca\(^{2+}\) into these stores is accomplished by a Ca\(^{2+}\) ATPase that is the target of action of thapsigargin. Thapsigargin irreversibly inhibits these ATPases with high selectivity over plasma membrane Ca\(^{2+}\) ATPases.\(^{22, 23}\) Interestingly, application of thapsigargin typically elevates [Ca\(^{2+}\)].\(^{24}\) This may initially be due to the prevention of ATPase-mediated Ca\(^{2+}\) buffering, but is ultimately the consequence of the activation of capacitative Ca\(^{2+}\) entry.\(^{24}\) This process involves activation of a plasma membrane channel that is believed to be triggered by the depletion of Ca\(^{2+}\) from reticular stores.\(^{24}\) This Ca\(^{2+}\) entry pathway also becomes apparent during prolonged application of agonists that mobilize inositol trisphosphate (IP\(_3\)), which is also associated with the depletion of reticular Ca\(^{2+}\) stores.\(^{26, 26}\)

There are two major pathways for Ca\(^{2+}\) release from endoplasmic reticulum. Both are multimeric ligand gated ion channels.\(^{27, 28}\) One is operated by IP\(_3\) (the IP\(_3\) receptor) while the other is usually considered to be gated by Ca\(^{2+}\) (to give Ca\(^{2+}\)-induced Ca\(^{2+}\) release, or CICR) in fact, both channels are gated by endo- and exogenous ligands (cyclic ADP ribose in the latter case).\(^{29}\) The activity of both channels is profoundly altered by physiological changes in [Ca\(^{2+}\)].\(^{27, 30, 31, 32, 33}\) Thus, both channels may be considered to be ligand gated, Ca\(^{2+}\) modulated release mechanisms. Rather fewer tools are available for modifying IP\(_3\) receptors in intact cells; heparin is an effective inhibitor, but does not readily gain access to the cytoplasm because of the abundance of negative charge in this polyanionic molecule.\(^{34}\) Rather more drugs are available to manipulate CICR. Ryanodine is a high affinity ligand for the CICR channel (which is also known as the ryanodine receptor), and promotes activation of the channel at low concentrations while blocking activity at high concentrations.\(^{30}\) In intact cells the unpredictable cell penetration of ryanodine can make it difficult to effectively titrate the desired effect, but robust concentrations enable CICR inhibition to occur quite readily. Dantrolene and 1,1′-diheptyl-4,4′-bipyridinium (DHBP) are also effective blockers of CICR, but are somewhat less potent than ryanodine. Studies of CICR are often complemented by the use of caffeine which, at millimolar concentrations, activates CICR in intact cells and triggers Ca\(^{2+}\) release when the stores contain Ca\(^{2+}\).\(^{35}\)

There is an emerging interest in the role of mitochondria in both apoptotic and necrotic cell injury. This organelle is potentially an important target, as well as an intracellular store, for Ca\(^{2+}\) in both physiological and pathophysiological settings. In addition to "sensing" cytoplasmic Ca\(^{2+}\) loads for the purpose of regulating ATP generation,\(^{36}\) mitochondria also act as a high-capacity, low affinity buffering mechanism.\(^{37}\) Once the mitochondrial Ca\(^{2+}\) set point is exceeded mitochondria essentially become a sink for Ca\(^{2+}\), and use the mitochondrial transmembrane potential to accumulate Ca\(^{2+}\) through the Ca\(^{2+}\) uniporter.\(^{38}\) There are two potential ways to modify mitochondrial Ca\(^{2+}\) uptake. Ruthenium red is a Ca\(^{2+}\) uniporter inhibitor, and presumably interacts directly with this as yet unidentified protein. This dye is actually a mixture of compounds, penetrates cells poorly, and lacks specificity for the uniporter because it inhibits CICR and VGCC at similar concentrations.\(^{39}\) It can also be difficult to use with fluorescent Ca\(^{2+}\) indicator dyes because it can quench fluorescence. A recent study described the properties of Ru360, which is the main active component of ruthenium red.\(^{40}\) This study demonstrated sub-nanomolar affinity of Ru360 for the uniporter in isolated mitochondria, and a high degree of specificity. However, the study also demonstrated that Ru360 is 104-fold less potent in intact cells, thus illustrating the difficulty of using such agents in cellular systems. The second way to block Ca\(^{2+}\) transport by the uniporter is by collapsing the membrane potential. This is rapidly and reversibly accomplished by protonophore uncouplers such as carbonyl cyanide 4-(trifluoromethoxy)phenyl hydrazone (FCCP) or carbonyl cyanide 3-chlorophenyl hydrazone (CCCP) (see, for example). Mitochondrial depolarization can also be triggered by blocking electron transport and ATP consumption with rotenone and oligomycin in combination, although these agents are not as readily reversed.\(^{42}\) Clearly, there is substantial potential for additional effects beyond blocking Ca\(^{2+}\) transport when using these drugs, and it may be important to control for plasma membrane depolarization or alterations in intracellular pH in these experiments.\(^{43}\)

Mitochondria represent a relatively transient storage mechanism for Ca\(^{2+}\), so that Ca\(^{2+}\) accumulated during a stimulus is typically released when the stimulus is removed. In this way mitochondria shape the recovery of [Ca\(^{2+}\)], elevations back to baseline, and may alter cell function as a consequence. Under physiological conditions the primary Ca\(^{2+}\) release mechanism is a Na\(^+\)/Ca\(^{2+}\) exchange mechanism on mitochondria.\(^{45}\) This process is pharmacologically distinct from the Na\(^+\)/Ca\(^{2+}\) exchanger in the plasma membrane. The diltiazem analogue CGP 37157 is an effective inhibitor of mitochondrial Na\(^+\)/Ca\(^{2+}\) exchange, and can be used to illustrate the role of mitochondrial Ca\(^{2+}\) release in shaping the [Ca\(^{2+}\)] transient.\(^{44, 45}\) When subjected to very large Ca\(^{2+}\) loads mitochondria can undergo a catastrophic alteration in permeability as a result of the activation of the permeability transition pore.\(^{46, 47}\) Activation of this non-selective ion channel collapses the membrane potential and can release Ca\(^{2+}\) both through the pore directly, and may also allow the Ca\(^{2+}\) efflux via reversal of the uniporter.\(^{48}\) Cyclosporin A is perhaps the most potent inhibitor of the permeability transition pore in intact cells, although its affinity for calcineurin can make results difficult to interpret. However, although the activation of the permeability transition pore has been extensively characterized in isolated mitochondria, the study of this phenomenon and its consequence for cellular Ca\(^{2+}\) homeostasis is still in its infancy.

### Ca\(^{2+}\) Binding Proteins

A careful analysis of the relationship between Ca\(^{2+}\) entry and the subsequent elevation in [Ca\(^{2+}\)] suggests that >99% of the Ca\(^{2+}\) that enters neurons does not appear as free Ca\(^{2+}\) in the cytoplasm.\(^{49}\) The cellular entity (or entities) that serves this endogenous buffer capacity has not been conclusively identified. However, it is clear that proteins that bind Ca\(^{2+}\) with high affinity do...
have the ability to act as short-term buffering mechanisms that modify brief Ca\textsuperscript{2+} transients. There is a large family of such proteins that may participate in Ca\textsuperscript{2+} buffering and signal transduction, including calmodulin, calbindin, and parvalbumin. Unfortunately it is difficult to pharmacologically manipulate the action of these proteins in an acute fashion, which has made it difficult to establish their actual physiological role. Several interesting suggestions have been made based on either over expression or knock out of members of the family of Ca\textsuperscript{2+} binding proteins. In general, over-expression of Ca\textsuperscript{2+} binding proteins diminishes the magnitude of brief Ca\textsuperscript{2+} transients, an effect that can be mimicked by introducing Ca\textsuperscript{2+} chelators into cells. The consequences of modifying these proteins for the long-term cellular activity is more difficult to predict. For example, one might suppose that augmenting Ca\textsuperscript{2+} binding protein expression would be protective for neurons, and conversely, that eliminating a Ca\textsuperscript{2+} buffering mechanism might be toxic to neurons. However, recent studies using a calbindin D28k knockout found protection against ischemic injury. Thus, although it is possible to use drugs to prevent the coupling between calmodulin and its effector pathways, the inability to acutely modify the Ca\textsuperscript{2+} buffering function of Ca\textsuperscript{2+} binding proteins will likely continue to make it difficult to fully understand the role of these proteins in Ca\textsuperscript{2+} homeostasis.

Clearly, for a cell to maintain viability Ca\textsuperscript{2+} influx must be balanced by Ca\textsuperscript{2+} efflux across the plasma membrane. In most cells this is accomplished by the actions of a Ca\textsuperscript{2+} ATPase and the plasma membrane form of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange. The ATPase is typically considered to be of high affinity but of limited capacity. This pump is relatively insensitive to thapsigargin and related agents. Indeed, there is a notable lack of effective inhibitors of the plasma membrane Ca\textsuperscript{2+} ATPase that can be used in intact cells. Although the pump also moves protons, pH modulation of ATPase activity is not an effective way of manipulating its activity because pH alterations also alter mitochondrial Ca\textsuperscript{2+} transport, and there are essentially no drugs that effectively block ATPase activity. The Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange is rather more amenable to manipulation in several ways. As the normal mode of operation allows the entry of 3 Na\textsuperscript{+} for the efflux of a single Ca\textsuperscript{2+} ion, removing extracellular Na\textsuperscript{+} effectively blocks exchanger activity. This can lead to several confounds that result from the inhibition of other Na\textsuperscript{+} dependent transporters, including the Na\textsuperscript{+}/H\textsuperscript{+} exchange, which results in intracellular acidification. Alternatively, a series of amiloride analogues has been developed that block exchanger activity with a modest amount of selectivity over other Na\textsuperscript{+} dependent transport processes. These analogues include benzamil and dichlorobenzamil. One of the more interesting aspects of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange is highlighted in light blue, while the gradients and stimuli that drive Ca\textsuperscript{2+} movements are indicated in white. The lefthand side of the diagram illustrates the processes that decrease [Ca\textsuperscript{2+}], while the righthand side shows the pathways that elevate cytosolic Ca\textsuperscript{2+}. The elongated ellipses represent endoplasmic reticulum; the short ellipses represent mitochondria. Abbreviations include: cADPR - cyclic ADP ribose; iCrac - the capacitative calcium entry current; L/VGCC - ligand/voltage gated calcium channel; RR - ruthenium red.
exchange is its reversibility.\textsuperscript{56, 58} A common feature of Na\textsuperscript{+} dependent transporters, the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange can run in reverse if the gradients that drive its operation change sufficiently. Thus, if cells are depolarized and Na\textsuperscript{+} loaded, the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange can operate as a Ca\textsuperscript{2+} entry pathway, the so-called ‘reverse mode’ of operation.\textsuperscript{59, 60} Perhaps surprisingly, drugs such as KB-R7943 preferentially block the reverse mode of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange preferentially over the regular mode.\textsuperscript{61, 62} These drugs then become useful tools to establish the potential contribution of reverse mode Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange as a Ca\textsuperscript{2+} influx pathway under various circumstances, and these studies have suggested a larger than anticipated contribution to neuronal Ca\textsuperscript{2+} responses triggered by a number of different stimuli.\textsuperscript{63}

It is clear that manipulating Ca\textsuperscript{2+} buffering presents a number of significant pharmacological challenges,\textsuperscript{39} even when the process is considered on a whole cell, essentially unidirectional basis. The reality is likely to be substantially more complex. The predominant buffering mechanisms will change as the duration or intensity of a stimulus is altered. Where small brief transients are buffered by cytoplasmic binding processes, a stimulus of longer duration will result in Ca\textsuperscript{2+} sequestration into endoplasmic reticulum, and in transport across the plasma membrane. Even larger Ca\textsuperscript{2+} loads that exceed the set point result in mitochondrial Ca\textsuperscript{2+} accumulation. However, it is rarely clear when and if clear boundaries exist between these processes at any given magnitude of Ca\textsuperscript{2+} load. Moreover, intracellular spatial and temporal limitations of Ca\textsuperscript{2+} signals can be accomplished by the juxtaposition of Ca\textsuperscript{2+} entry, release and uptake processes, so that signaling from one Ca\textsuperscript{2+} storage compartment to another may exist with relatively little impact on overall cytoplasmic free Ca\textsuperscript{2+} concentrations.\textsuperscript{64, 65, 66, 67} Thus, while the drugs described here are potentially of great value in dissecting Ca\textsuperscript{2+} homeostasis mechanisms, it is important to carefully consider the limitations in the information that can be obtained from their use.

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**References**

44. Cox and Matlib (1993) TIPS 14 408.
# Calcium Signaling Agents available from Tocris

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**Calcium ATPase Modulators**

| 1236 BHQ | Inhibits SERCA ATPase |
| 1235 Cyclopiazonic acid | Inhibitor of SERCA ATPase |
| 1138 Thapsigargin | Potent inhibitor of SERCA ATPase |

**Calcium Sensitive Protease Modulators**

| 0448 Calpeptin | Calpain and cathepsin L inhibitor |

**General Calcium Signaling Agents**

| 1234 A23187, free acid | Calcium ionophore |
| 1114 CGP 37157 | Antagonist of mitochondrial Na+·Ca2+ |
| 0600 Nimodipine | Less active enantiomer of (1123) |
| 0601 Nifedipine | Ca2+ channel blocker (L-type) |
| 0604 Verapamil | Ca2+ channel blocker (L-type) |
| 0840 Loperamide | Ca2+ channel blocker (HVA) (L/N-type) |
| 1085 α-Conotoxin GVIA | Ca2+ channel blocker (N-type) |
| 1084 α-Conotoxin MVIIC | Ca2+ channel blocker (N, P and Q-type) |

**Calcium Channel Modulators**

| 0685 Diltiazem | Ca2+ channel blocker (L-type) |
| 1075 Nifedipine | Ca2+ channel blocker (L-type) |
| 1124 (R)-(+)-Niguldipine | Less active enantiomer of (1123) |
| 1123 (S)-(+)-Niguldipine | Ca2+ channel blocker (L-type) |
| 1124 (R)-(+)-Niguldipine | Ca2+ channel blocker (L-type) |
| 1085 Loperamide | Ca2+ channel blocker (HVA) (L/N-type) |
| 1084 α-Conotoxin GVIA | Ca2+ channel blocker (N-type) |
| 0452 CCCP | Oxidative phosphorylation uncoupler |
| 1101 Cyclosporin A | Calcineurin inhibitor |
| 0453 FCCP | Oxidative phosphorylation uncoupler |
| 0479 Malonoben | Oxidative phosphorylation uncoupler |
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