Molecular Neurobiology

ISSN0893-7648/98/24(1–3): 65–86/$15.50

Pharmacological Modulation of Intracellular Ca^{2+} Channels at the Single-Channel Level

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Abstract

Synaptic signaling, memory formation, neuronal development, and neuronal pathology are strongly influenced by the properties of intracellular Ca^{2+} channels, ryanodine, and inositol 1, 4, 5 trisphosphate receptors. This review will focus on recently developed and discovered pharmacological tools to modulate these channel proteins at the single-channel level. It will allow the readers of *Molecular Neurobiology* to evaluate the current knowledge on the pharmacological modulation of intracellular Ca^{2+} channels and to direct future research efforts effectively using available experimental tools and concepts.

Index Entries: Ryanodine receptor; inositol 1, 4, 5 trisphosphate (IP_3) receptors; sarcoplasmic reticulum; endoplasmic reticulum; intracellular Ca^{2+} signaling; cytosol.

Introduction

Since the initial discovery and characterization of intracellular Ca^{2+} channels, their importance for the function of neurons, signal transduction, and information processing has been recognized *(1–9)*. Recent studies show that intracellular Ca^{2+} channels are crucial components of diverse processes such as learning and memory formation as well as neuronal differentiation, neurogenesis, and apoptosis *(10–17)*. The important neuron-in-neuron concept developed by Berridge and co-workers *(17)* is one prominent way to explain a number of functions of intracellular Ca^{2+} channels in neurons. To understand fully the mechanism of action of intracellular Ca^{2+} channels as part of neuronal Ca^{2+} signaling, it is necessary to analyze the molecular function of these trans-membrane proteins at the single-channel level. To this end, it is of great importance to understand drug-induced modulation of these channels and what pharmacological tools are available to investigate their role in neuronal physiology.

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Therefore, this article will review published scientific data describing the effects of currently available chemical compounds on intracellular Ca^{2+} channels, primarily at the single-channel level. This appears to be not only timely but also necessary for two reasons: First, the number of published data involving intracellular Ca^{2+} channels in neuronal function and physiology has increased tremendously. However, the described effects are not fully understood in all cases and require careful analysis using molecular neurobiological methods. Second, the number of studies describing pharmacological properties of intracellular Ca^{2+} channels has grown in parallel. However, a number of these studies have been performed with non-neuronal cells, especially muscle and epithelial cells and also using, by means of molecular biology, artificially expressed isoforms of intracellular Ca^{2+} channels. The importance and the impact of these data on the study of neuronal function and in molecular neurobiology is not always immediately evident.

To date, two intracellular Ca^{2+} channels have been described. Both the inositol 1, 4, 5-trisphosphate receptors (IP_3R) and the ryanodine receptor (RyR) are exclusively expressed in intracellular membranes, particularly the endoplasmic reticulum (ER) membrane. These proteins each form tetrameric complexes and share substantial sequence homology in their functional domains *(7).* For both types a number of molecularly and physiologically distinct isoforms and splice variants are known. Despite the fact that biophysical data for specific isoforms and the localization of individual subtypes are available *(8,9,18–21),* isoform-specific agonists or antagonists have not been established. The number of physiological agents modulating intracellular Ca^{2+} channels present in the cytosol or the lumen of the ER is limited. Recent reviews and reports have summarized the importance of endogenous ligands of both the RyR *(22–25)* and the IP_3R *(20),* such as ATP, Ca^{2+}, cADPR, IP_3, and lipophilic-messenger substances including arachidonic acid and leukotriene B4. In brief, the activity of both the RyR and the IP3R are strongly dependent on the level of cytosolic free Ca^{2+} *(26,27),* the presence of ATP *(3,28),* and the concentration of Ca^{2+} in the ER lumen *(24,29).* In contrast to the RyR, which can be active in the presence of adequate amounts of cytosolic free Ca^{2+} alone, the IP_3R is a truly ligand-gated Ca^{2+} channel. Both cytosolic free Ca^{2+} and IP_3, generated by the activity of phospholipase C, are necessary for the activation of the IP_3R *(26).* Subsequently the effects and, if available, the mechanism of action of agonists and antagonists characterized using single-channel recordings will be reviewed, first for the RyR and then for the IP_3R.

Modulation of RyR Channel Activity

RyR (Ca^{2+}-induced Ca^{2+} release channels) are essential components in intracellular Ca^{2+} signaling for most cell types, including neurons *(13,15–17).* These large tetrameric channel proteins are homologous to, but physiologically different from, IP_3R (*see* next section). Typically RyR channels display a bell-shaped activity dependence for the cytosolic Ca^{2+} concentration similar to type 1 IP_3R. Lack of activation by low, and inhibition by high cytosolic Ca^{2+} concentrations tunes RyR activity to a narrow, physiologically relevant range *(7,26,30–33).* However, the various RyR isoforms display differences in the width of this physiological range of Ca^{2+} concentrations that already activate but do not inhibit the RyR. This leads to tissue- and cell-specific distributions of isoforms most suited for particular physiological tasks *(21).* RyRs are characterized by selectivity for cations paired with a low selectivity among cations, voltage-independent channel activity, and physiologically relevant interactions with a number of intracellular proteins *(34)* and signaling substances, such as cADPR *(25),* arachidonic acid and its derivatives *(23),* sphingolipids *(35–37),* and ATP *(28,38).*

Ryanodine Effects on the RyR

The alkaloid ryanodine binds specifically to the RyR and has been used extensively in

radioligand-binding studies *(2–4).* Ryanodine binds to the RyR channel in its open state, stabilizes the open state, and blocks the inhibitory action of compounds such as ruthenium red (*see* subsequent paragraph on highly charged molecules). The activity of ryanodine to stabilize the open state of the RyR channel is accompanied by a change in the gating mode. Micromolar concentrations of ryanodine induce sublevel gating modes with long open conductances *(2,5,6,39–41).* This effect has been shown to be stronger with a higher stabilization of the channel open state for the skeletal than for the cardiac RyR *(6).* The action of ryanodine on the RyR can not be easily categorized as a plain inhibitor of channel activity. However, the compound is still of great importance for experiments that try to identify the nature of Ca^{2+} release channels due to ryanodine's high binding specificity.

Caffeine Activation of RyR

Caffeine is one of the most widely used tools to investigate RyR-mediated Ca^{2+} signaling *(40,42).* Submillimolar concentrations of caffeine affect the frequency of channel openings and leave the mean open times unchanged, thus leading to an increased open probability of RyR channels *(43).* Others could show that in addition to the rise in frequency also the duration of channel open events increased *(44).* Caffeine-activated channels show an identical behavior with respect to pharmacological modulation when compared to the Ca^{2+} activated RyR channel *(43).* The activating effect of caffeine at submillimolar concentrations is dependent on a minimal activating concentration of cytosolic-free Ca^{2+} of around 0.1 μ*M.* However, increasing the caffeine concentration to 5 m*M* or higher leads to a Ca^{2+}-independent RyR channel activation *(43).* This property combined with its receptor specificity and its well-characterized mechanism of action make caffeine an important tool for the analysis of RyR function.

Indirect Modulation of the RyR by Immunosuppressants Through the Associated Protein FKBP

FK506-binding protein (FKBP) is associated with the RyR and occurs naturally as part of the RyR channel complex of both the cardiac and skeletal type *(45–46).* FKBP stabilizes RyR channel function by increasing the number of full-conductance channel openings and increasing channel mean open times *(47).* The immunosuppressant drugs FK506 and rapamycin disrupt the FKBP RyR interaction by binding to FKBP and dissociating it from the RyR channel complex *(47–50).* Treatment of RyR with micromolar concentrations of FK506 or rapamycin abolishes the stabilizing effect of FKBP on RyR leading to reduced channel current amplitudes, a lower open probability for full-conductance channel openings, and a higher open probability for substate channel openings *(47,48,50).* FK506 and rapamycin are useful tools to evaluate the single-channel properties of RyR as well as the quality of RyR preparations. The use of these compounds allows one to analyze the contribution of accessory proteins to RyR channel function. Additionally, the effect of FK506- or rapamycin-sensitive accessory proteins on the phosphorylation state of the RyR channel and the contribution of such proteins to the function of the RyR complex can be investigated.

Effect of Highly Charged Molecules on the RyR

The effects of a number of highly charged compounds on RyRs has been tested. RyRs being Ca^{2+}-activated Ca^{2+} channels rely on the negative charge of the receptor complex and especially of their Ca^{2+} binding sites to attract Ca^{2+} ions for receptor activation *(51).* The fixed negative surface charge of the RyR potentiates conduction and brings about divalent cation selectivity of the channel *(52).* Charged substances that are able to bind to the RyR and thereby change the local negative charge of the protein complex, can potentially modulate

channel activity. Polyanions, molecules that are highly negatively charged, such as heparin, pentosan polysulfate, or polyvinyl sulfate, increase RyR channel activity in a Ca^{2+}-dependent manner. Activation of the channel occurs only when the cytosolic free Ca^{2+} concentration is higher than 20 nM. Half-maximal activation was observed at heparin concentrations of 230 ng/mL or pentosan polysulfate concentrations of 162 µg/mL *(39).* An increase in the local charge of the receptor complex towards more negative values by binding of heparin would attract more Ca^{2+} ions. This localized increase in the Ca^{2+} concentration would thus lead to higher activation of channels. For a highly positively charged compound, protamine, the opposite effect has been described *(53).* Protamines *(54)* completely inhibit RyR channel activity with a half-maximal inhibition of 37 µg/mL. Inhibition of RyR channel activity is Ca^{2+} independent and complete at all physiologically relevant Ca^{2+} concentrations at protamine concentrations of 40 µg/mL or higher *(53).* Binding several molecules of protamine to the RyR as indicated by the Hill coefficient presumably adds positive charges to the channel complex and prevents binding of Ca^{2+}. A lack of activating Ca^{2+} ions binding to the receptor would consequently prevent channel activity.

A group of polycationic compounds, containing ruthenium red as its best known representative, is known to affect a number of physiological processes *(55)* and was shown to be an effective blocker for RyR channels *(56).* Ruthenium red and the related compounds tetraamine palladium and tetraamine platinum block RyRs when applied from the cytoplasmic side at submicromolar concentrations. As with polyanions and protamines, Hill coefficients indicate the involvement of several molecules in the inhibitory mechanism of action of these compounds. Owing to their polycationic nature, stronger positive holding potentials increase the inhibitory effect presumably by providing a higher electrical driving force of the compound into the channel *(56).* With ruthenium red showing the highest affinity of RyR in its group, it is widely used in physiological studies to test the function of RyR. It is also used frequently to evaluate the effects of other signaling pathways not affected by ruthenium red, especially the role of the IP_3R under RyR block *(23,57).*

Polyamines, a group of naturally occurring cationic compounds, including spermine, spermidine, and putrescine have been shown to be blockers of the RyR channel with half-block concentrations in the sub-millimolar range. The blockade is dependent on the holding potential, current carrier, and current direction, indicating that polyamines are permeable blockers that might interact with the cations carrying the current in the channel *(58).*

Modulation of RyR Channels by Nitric Oxide

Both the skeletal and cardiac RyR are inhibited by nitric oxide (NO) at the single-channel level. NO reversibly decreases the open probability of the RyR channel *(59,60).* However, under different experimental conditions, NO and related compounds were found to activate RyR channels *(61,62).* The measured effects depend crucially on the oxidation state of the RyR and the NO-generating or NO-related compound *(63).* Besides the obvious physiological roles or NO-induced modulation of RyR channel activity (changes in contractility and excitability) the inhibitory effect of NO can be used as a pharmacological tool and has to be taken into account, especially when other NO-induced processes are being investigated.

Scorpion Toxins Can Serve as Selective RyR1 Channel Modulators and as Molecular Probes to Investigate the Structure of the RyR

Imperatoxin A, a peptide produced by and found in the venom of the scorpion *Pandinus imperator,* acts as an activator of RyR channel activity by stabilizing a subconductance state. It shows sequence similarity to a portion of the skeletal muscle dihydropyridine receptor that

activates RyR1 through protein-protein interaction *(64–71).* Imperatoxin A selectively activates RyR1 and not RyR2 further corroborating the hypothesis that mimicking the activating domain of the dihydropyridine receptor is the underlying mechanism of action *(64).* This sequence similarity to the interacting domain of the skeletal muscle dihydropyridine receptor is also conserved in maurocalcin *(72)* suggesting a similar mechanism of action. Maurocalcin is a peptide from the venom of scorpion *Scorpio maurus* and shows 82% sequence identity with imperatoxin A. The peptide induces long-lived subconductance states in RyR1, reversibly binds to the RyR at a different site than the ryanodine binding site and leads to an increase in Ca^{2+} release by increasing the net activity of the RyR channel *(72).* Imperatoxin I, a toxin found in vivo together with imperatoxin A in the scorpion *Pandinus imperator* blocks openings of both the skeletal and cardiac RyR reducing the release of Ca^{2+} by RyR *(64).* However, the toxin presumably acts indirectly on the RyR by activating phospholipase A2. Presumably lipid products of phospholipase A2 subsequently inhibit RyR channel activity *(67).* Ryanotoxin, a peptide from the venom of the scorpion *Buthotus judiacus* was found to induce a substate conductance with long mean open times in type 1 RyR channels. Despite its eponymous effect on the RyR similar to ryanodine it does not bind to the ryanodine binding site similar to imperatoxin A and maurocalcine *(73).* A genetic homology to imperatoxin A seems likely, but is not proven. With the complete sequence of both maurocalcine and imperatoxin A published *(68,72),* selective tools for the activation of RyR1 and not RyR2 through the stabilization of channel subconductance behavior are available.

Niflumic Acid Has Differential Effects on RyR Isoforms

Besides its effects on anion transport and chloride channels, niflumic acid has been shown to have multiple concentration-dependent and subtype-specific effects on RyR *(74,75).* Whereas niflumic acid did not change the pharmacology, unitary conductance and reversal potential of RyR channels, a differential effect on the open probability of different RyR channel types was observed. The α isoform (homologous to mammalian RyR1 *[21]*) shows an increase in the channel open probability and mean open time at 10 μ*M* niflumic acid, which are reduced by further increasing the niflumic acid concentration to 100 μ*M* thus producing a bell-shaped concentration dependence. The open probability and mean open time of the β isoform channel (homologous to mammalian RyR3; similarities to RyR2 *[21]*) are increased with a sigmoidal activation curve by higher concentrations of niflumic acid *(74,75).* Niflumic acid can therefore provide a useful tool to differentially modulate different RyR isoforms, a property that most other modulators of RyR function do not have.

Dantrolene Modulates the RyR Channel at Two Binding Sites

Dantrolene, a widely used muscle relaxant, binds specifically to the RyR as shown by affinity-labeling studies *(76,77)* and inhibits RyR-mediated Ca^{2+} release *(78).* At micromolar concentrations, it binds to a low-affinity binding site of the RyR and reduces channel activity. However, when applied at nanomolar concentrations, dantrolene binds to a high-affinity binding site of the RyR and increases the channel open probability and mean open time *(79).* Besides its widespread use to block the RyR channel at high concentrations, dantrolene has the potential to differentially activate the same channel at low concentrations, thus being an interesting tool to assess RyR function.

Modulation of RyR Activity by 2,3-butanedione Monoxime

2,3-butanedione monoxime is a widely used inhibitor of muscle contraction and affects both skeletal and cardiac muscles *(80).* At the single channel level 2,3-butanedione monoxime increases the open probability of both cardiac and skeletal RyRs at free cytosolic Ca^{2+} concen-

trations of 0.5–1 μ*M*. Whereas channel conductances are unaffected by 2,3-butanedione monoxime, the number of channel openings is larger, with increased concentrations of 2,3-butanedione monoxime present on either the luminal or the cytoplasmic side of the channel *(81)*. At higher cytosolic free Ca^{2+} concentrations skeletal RyR channels are inhibited by 2,3-butanedione monoxime (IC_{50} approx 2.5 m*M*), while the cardiac RyR is unaffected. The single channel data are corroborated using RyR population [^{3}H]ryanodine binding assays *(81)*. This makes 2,3-butanedione monoxime a very versatile tool to modulate channel activity depending on the RyR-subtype and the cytosolic free Ca^{2+} concentration.

The Effect of Anesthetics on RyR Channel Activity

Several studies indicate that anesthetics such as tetracaine, dibucaine, benzocaine, procaine, lidocaine, QX-314, prilocaine, and bupivacaine specifically bind to the RyR *(82–84)*. Single-channel analysis of the interaction showed that tetracaine and procaine reduces channel activity, and QX-314 application leads to a fast block of the RyR channel supporting the findings of specific low- and high-affinity binding sites on the RyR. In addition, all three substances induce a voltage-dependent block of the RyR channel with long open subconductance states *(85)*. Application of the β-adrenergic receptor blocker propanolol to RyR channels blocks their activity completely. Activity could only be regained to a subconductance state by increasing ATP concentration on the cytosolic side of the channel *(86)*. The described anesthetics can be useful to investigate RyR function, but their presence due to experimental requirements has to be taken into account when investigating other effects involving the RyR.

Chemical Modification as a Tool to Modulate RyR Activity

As with many other ion channels *(87)* a number of chemicals were described that modulate RyR function. In general highly reactive and moiety-specific substances are used to investigate channel subunit composition, channel pore size, ion specificity, and other biophysical parameters related to the composition of functionally important channel domains and moieties.

Hydrophilic methanethiosulfonate derivatives (MTSEA, MMTS) applied to the cytosolic side of the open RyR channel covalently modify RyR, presumably inside the conducting channel pore. Methanethiosulfonate derivatives decrease channel-current amplitude in discrete steps until a complete block is achieved *(41)*. Dwell times, open probability, and modulation by ATP, Ca^{2+}, and caffeine are not changed by methanethiosulfonate compounds, indicating a highly specific interaction of the sulfhydryl compounds with cysteine residues in the channel pore region *(41)*.

Oxidizing reagents such as 4,4′-dithiodipyridine and thimerosal mediate changes in channel dwell time *(88)*. Transient activation followed by inhibition of RyR was explained by a number of differently affected cysteine-containing domains on RyR *(88–92)*. Modification of RyR by sulfhydryl oxidation using 2,2′-dithiodipyridine resulted in a twofold effect *(93)*. Firstly, channel activity was increased and could specifically be decreased by addition of reducing glutathione. A similar effect was observed using 4,4′-dithiodipyridine as the activating sulfhydryl (SH) oxidation reagent and dithiothreitol (DTT) as the reducing agent *(94)*. Secondly, a broadening of the bell-shaped activity dependence on cytosolic free Ca^{2+} by SH oxidation was observed *(93)*.

An increased open probability of the RyR channel is also observed after addition of hydroxyl radicals to the cytosolic side of the channel. Altogether, reagents interacting with SH groups of the RyR prove to be an excellent tool to modulate a variety of biophysical parameters of the channel.

The polysulfonated napthylurea suramin reversibly increases the mean open time and conductance of both type 1 and 2 RyR channels and thus also increases the channel open probability *(95)*. Despite its chemical nature, suramin does not oxidize SH groups of the

Table 1
Summary of Effects of Pharmacological Modulators on the RyR

Drug	Site of action	Dose(s)	References
Ryanodine	Pore region	Kd = 9.0 nM 1–10 μ*M*	*(2–4,1–41)*
Caffeine	Not determined	<1 m*M:* Ca^{2+} independent activation; > 5 m*M:* Ca^{2+} independent activation	*(43)*
FK506, rapamycin	FKBP	Micromolar	*(47–50)*
Heparin	Not determined	EC_{50} = 230 ng/mL	*(39)*
Pentosan polysulfate	Not determined	EC_{50} = 162 μg/mL	*(39)*
Protamine	Not determined	EC_{50} = 37 μg/mL	*(53)*
Ruthenium Red	Channel pore (?)	Submicromolar	*(56)*
Polyamines	Not determined	Submillimolar EC_{50}	*(58)*
Imperatoxin A, Maurocalcin, ryanotoxin	Bind to dihydro-pyridine binding site (Imperatoxin A; others ?)	Kd = 11–13 nM 10–50 nM	*(64,71–73)*
Niflumic acid	Not determined	10 μ*M:* activating 100 μ*M:* inhibiting	*(74,75)*
Dantrolene	Not determined	Nanomolar: activating; micromolar: inhibiting	*(79)*
2,3-butanedione monoxime	Not determined	[Ca^{2+}] and subtype dependent	*(81)*
tetracaine	Not determined	Two-fold decrease: 150 μ*M*	*(85)*
procaine	Not determined	Two fold decrease: 4 m*M*	*(85)*
QX314	Not determined	block: millimolar	*(85)*

RyR channels. The effects seem to be due to the binding of suramin to a binding site on the RyR different from the ATP binding site *(95).*

Also a number of studies investigating the modification of specific amino acids of the RyR by reactive chemicals found highly specific effects on RyR channel properties. Fluorescein isothiocyanate (FITC; modifies RyR lysine residues) was found to increase single-channel open probability by activating the channel to a 60% subconductance state. FITC also decreased ryanodine binding to the RyR significantly *(96).* Similarly 7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole, modifying RyR lysine, tyrosine and cysteine residues, change RyR channel gating properties with time-dependent activation followed by inactivation *(97).* Probably using a similar mechanism of action, inhibiting and activating compounds were identified. Whereas diethyl pyrocarbonate (modifies histidine residues) and disulfonic stilbene derivatives such as 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (modify amino residue in the gating domain) induced longer open states of the channel and activated RyRs *(98–100),* a number of chemically reactive compounds inhibited channel activity. Dicyclohexylcarbodiimide (modifies carboxy residues) and dinitrofluorobenzene (forms dinitrophenyl derivatives with amines) inhibits ryanodine binding and blocks channel openings *(101–103).* However, dinitrofluorobenzene-mediated block of RyRs can also be converted to re-activation of the channel using submicromolar concentrations of ryanodine and low free cytosolic Ca^{2+} concentrations under certain incubation conditions of RyRs with dinitrofluorobenzene *(103).*

Overall, the use of different, low molecular-weight, moiety-modifying compounds on the RyR allows selective modification of specific

RyR channel properties, and the fine-tuning of channel activity according to experimental requirements.

Modulation of IP_3R Channel Activity

External signals such as neurotransmitter substances or hormones, bind to, and activate, G-protein coupled receptors or tyrosine-kinase linked receptors in the plasma membrane *(104).* Receptor activation stimulates phospholipase C to hydrolyze the membrane-associated lipid phosphatidyl inositol (4,5) bis-phosphate (PIP_2), producing the second messengers IP_3 and 1,2-Diacyl glycerol. IP_3 diffuses through the cytosol and binds to IP_3Rs on the surface of the ER, the Ca^{2+} store of the cell, whereupon it mobilizes intracellular Ca^{2+} release. The IP_3R consists of more than 2700 amino acid residues and is highly conserved among species *(105–114).*

The IP_3R contains at least three functionally distinct regions: a cytosolically located IP_3 binding domain, a hydrophobic membrane-spanning region forming the ion-channel pore, and a large regulatory domain linking the two. The pore-forming region is highly conserved and even exhibits homology with the RyR *(7).* The IP_3 binding site *(105,115)* is located in the N-terminal region of the receptor and contains a number of charged residues that are apparently necessary for IP_3 binding *(116).* The remaining bulk of the receptor, which is also located in the cytosol, is designated the regulatory domain, and couples the binding site to the pore. Several putative regulatory sites are found in this domain including those for phosphorylation *(105,107,108),* ATP binding *(117),* and Ca^{2+} binding *(118,119).* In addition, there are sites for interactions with accessory proteins, such as the Ca^{2+} binding protein calmodulin *(120)* and the immunophilin FK506 binding protein (FKBP) *(121).*

Following characterization of what is now designated the type I IP_3R, two additional isoforms have been identified: types II and III *(106,122,123).* The three IP_3R isoforms are 60–70% homologous with one another *(106,122)* and vary in their tissue distribution *(105,106,122–125).* Each receptor subtype exhibits different patterns of IP_3-induced Ca^{2+} release: Ca^{2+} oscillations can occur via the type I receptor, whereas larger, sustained signals are seen from types II and III *(57,126).*

These IP_3-induced Ca^{2+} signals are responsible for a wide variety of cellular processes, including secretion, gene expression, metabolism, contraction, cell death, cell proliferation, and neuronal excitability. Modulation of IP_3-induced Ca^{2+} signaling can be achieved by the use of specific pharmacological regulators. The effects of these regulators have been investigated, largely at the single-channel level, and will now be discussed further.

Inositol Phosphates and Their Analogues

Certain inositol phosphates, namely D-*myo* IP_3, the D-isomer of $I(2,4,5)P_3$ and $I(4,5)P_2$, possess structural features which are essential in intracellular Ca^{2+} release via the IP_3R. All of these compounds have a pair of vicinal phosphates at the 4- and 5- positions, apparently crucial for full Ca^{2+} mobilization, with additional phosphates in positions 1 or 2 increasing receptor binding specificity (as reviewed in ref. *127*). The enantiomer of D-*myo* IP_3, L-IP_3, has been shown to be inactive as well as $I(1,3,5)P_3$ (reviewed in ref. *127*), indicating the importance of the 4-position and its orientation. The metabolic products of IP_3: $I(1,4)P_2$ and I(1,3,4,5) tetrakisphosphate are found to be some 1000-fold less potent. Inositol phosphates and synthetic analogues of IP_3 have revealed a great deal of pharmacological information about the IP_3 binding site *(128–135).* D-*myo* IP3 is the naturally occurring isomer and has maximal potency ($EC_{50} \approx 0.12$ μ*M*), which is some 10-fold higher than that of its nonhydrolyzable analogue, $I(2,4,5)P_3$ ($EC_{50} \approx 1.2$ μ*M*), and 200–400 fold greater than $I(1,4)P_2$ ($EC_{50} \approx 20$–50 μ*M*) *(136–138).* Substitution, either partially or totally, of the phosphate groups by bulky phosphorothioates produces high affinity ligands, which are resistant to the enzymes that degrade IP_3 in the cellular environment (the IP_3 3-kinase and 5-phosphatase) *(129,139,140).*

Table 2
Summary of Effects of Pharmacological Modulators on the IP3R

Drug	Site of action	Dose(s)	References
D-*myo* I(1,4,5)P_3	I(1,4,5)P_3 binding site (N-terminal 600 amino acids)	$EC_{50} \approx 0.12$ μ*M*	*(136–138)*
I(2,4,5)P_3	I(1,4,5)P_3 binding site (N-terminal 600 amino acids	$EC_{50} \approx 1.2$ μ*M*	*(136–138)*
I(1,4)P_2	I(1,4,5)P_3 binding site (N-terminal 600 amino acids	$EC_{50} \approx 20–50$ μ*M*	*(136–138)*
Adenophostins	I(1,4,5)P_3 binding site (N-terminal 600 amino acids	$K_i = 0.18$ n*M*	*(146)*
Thimerosal	Not determined	10–100 μ*M*	*(160)*
Ruthenium Red	Not determined	30 μ*M*	*(165)*
Caffeine	Not determined	5–50 m*M*	*(167,168)*
Aliphatic alcohols	Not determined	1–3%(v/v)	*(169,170)*
Polyamines	Not determined	1 m*M*	*(171)*
FK506	Not determined	$EC_{50} \approx 10–100$ nM	*(121,173)*
Heparin	I(1,4,5)P_3 binding site (N-terminal 600 amino acids)	10–100 μg/mL	*(28,31)*
Xestospongin C	Not determined	$IC_{50} = 358$ nM	*(182)*
2-Aminoethoxy diphenyl borate (2APB)	Not determined	$IC_{50} = 42$ μ*M* [for I(1,4,5) P_3 (100 nM)-induced Ca^{2+} release]	*(184)*

Thus, interaction with the IP_3R appeared to be limited to IP_3, a few inositol phosphates *(132,133),* some synthetic analogs *(128),* and the antagonists heparin *(141–143),* and decavanadate *(144–145);* that is, until the discovery of adenophostins *(146).* Adenophostins A and B are metabolites of the fungus *Penicillium brevicompactum* and were found to act as potent agonists of the IP_3R. Despite striking structural differences from IP_3, adenophostins were found to inhibit [^{3}H] IP_3 binding more potently than unlabeled IP3 *(146).* K_i values for adenophostins were calculated to be 0.18 nM, compared to the K_i for IP_3, which was 15 nM. Adenophostins, even at concentrations of 1 nM, were still found to produce significant Ca^{2+} release from cerebellar microsomes *(146).* Adenophostins are resistant to IP_3-metabolizing enzymes although their action is inhibited by heparin *(146).* Since the discovery of these compounds, synthetic analogs have been developed *(147–150).* 2-hydroxyethyl-α-D-glucopyranoside-2,3′,4′-trisphosphate is one such IP_3/adenophostin A analog. Investigations demonstrated that it was poorly metabolized by the 3-kinase and 5-phosphatase and was found to be considerably weaker than adenophostin A (some 1000-fold weaker as an agonist), indicating that the partial excision of the adenosine residue removed structural motifs that participated favorably in binding to the IP_3R *(147,151).* Although these compounds are not physiological regulators of the IP_3R in animal cells, they do substantiate earlier claims about the interaction of IP_3 with its receptor. The 3′ and 4′ equatorial diphosphates on the glucose ring of adenophostins, for example, can be superimposed on the 5′ and 4′ equatorial diphosphates respectively of IP_3. This concurs with earlier findings that the vicinal 4- and 5- phosphates are vital for I(1,4,5) P_3 activity, whereas the 1′ phosphate is less important. Additionally, it may be that the 2′ phosphate of

adenophostins is placed to fit the IP_3R more effectively than the 1′ phosphate of IP_3. Further investigations using nuclear Overhauser effect and molecular mechanics put forward the idea that adenophostin A owes its exceptional potency to an optimal spatial arrangement of the three phosphoryl groups coupled with the interaction of the adenine moiety and the putative IP_3 binding site *(151).*

Sulfhydryl Reagents

In many cell types, sulfhydryl reagents such as thimerosal (TMS) and oxidized glutathione (GSSG), have been shown to trigger Ca^{2+} oscillations *(152–153).* Monoclonal antibodies directed against the C-terminus of the IP_3R, inhibit these oscillations, thereby implicating the involvement of the IP_3 *(154–155).* Later studies found that TMS and GSSG increased the affinity of the receptor for IP_3 *(156)* and sensitized IP_3-induced Ca^{2+} release *(157)* although other findings indicated an inhibitory effect *(158).* Further studies concluded that at low TMS concentrations, IP_3-induced Ca^{2+} release from cerebellar microsomes was sensitized, and at higher concentrations it was inhibitory *(159).* The solubilized purified IP_3R from rat cerebellum was reconstituted into planar lipid bilayer membranes. In the presence of 10 μ*M* IP_3, 50 μ*M* ATP, and 0.2 μ*M* Ca^{2+}, the IP_3R channel opened to a conductance level of 53pS. In the presence of 100 μ*M* thimerosal (TMS), however, three higher-conductance levels (60pS, 80pS, and 120pS) were observed *(160).* More than one population of mean open times was found, both in the absence and presence of TMS. TMS decreased the length of the short opening significantly from 4.05 ms to 2.78 ms and increased the longer open time from 27.8 ms to 94.8 ms, resulting in an overall 10% increase in channel open time *(160).* TMS enhanced IP_3-induced Ca^{2+} release by both altering the open times of the channel significantly and causing a shift to higher conductance levels *(160).* TMS has been shown not to interfere with IP_3 binding *(161)* therefore it is unlikely that the site of action is at the IP_3-binding domain. An effect of TMS has been observed with RyRs also *(90,162),* which do not respond to IP_3 and thus have major differences in ligand recognition and coupling domains, strongly indicating that TMS acts at the Ca^{2+} channel region. Two cysteine groups at positions 2610 and 2613 are located in this area of the type 1 IP_3R, present in a TXCFICG motif, which is highly conserved in all three isoforms as well as RyRs. As TMS is a thiol-oxidizing reagent, these cysteine groups are the likely target sites for channel modification by TMS and other SH-oxidizing reagents involved in endogenous oxidative-reductive process. The redox state of the cell may therefore play a regulatory role with respect to the IP_3R.

Ruthenium Red

The polycationic dye, ruthenium red, generally used as an inhibitor of mitochondrial Ca^{2+} uptake, has been reported to inhibit Ca^{2+}-induced Ca^{2+} release, and at higher concentrations, inhibits ATPase activity (as summarized in ref. *55*). The general consensus with regards to using ruthenium red while recording IP_3-receptors, however, seems to be that it has no effect *(31),* although some reports indicate otherwise *(55,163–165).* In permeabilized rabbit pancreatic acinar cells, ruthenium red selectively depletes IP_3-sensitive Ca^{2+} stores, reducing their size, although the dye may be acting on another Ca^{2+}-release channel and not necessarily the IP_3R *(55).* IP_3R in carp olfactory cilia are also found to be inhibited by ruthenium red at the single-channel level *(165).* Investigation of this olfactory IP_3R was carried out using a large panel of antibodies raised against the three IP_3R isoforms. These results concluded that in carp olfactory cilia, an IP3-dependent channel is present, although it is distinct from the classical isoforms, which have already been characterized. This further raises interest as the RyR is inhibited by ruthenium red, and it has some homology with the classical IP_3R, although none of these are sensitive to the compound. Ruthenium red may therefore prove to be a useful tool in investigating this olfactory IP_3R isoform and discriminating between RyR and IP_3R.

Caffeine

Studies involving *Xenopus* oocytes illustrated that caffeine had no effect on the initial transient Ca^{2+} release induced by IP_3, although subsequent oscillations were completely abolished *(166).* This indicates that caffeine affects Ca^{2+}-induced Ca^{2+} release rather than IP_3-induced Ca^{2+} release. Further studies from rat cerebellum, have shown that caffeine (50 m*M*) inhibits the opening of the IP_3R and shifts the IC_{50} value (from 900 n*M* to 3.9 μ*M* at 50 m*M* Caffeine) such that more IP_3 is required for Ca^{2+} release *(167).* However, caffeine does not affect the total Ca^{2+} release at higher IP_3 concentrations or [^{3}H] IP_3 binding *(167).*

Caffeine-induced inhibition of the IP_3R has also been observed in single-channel experiments *(168).* Caffeine inhibited the activity of the channel in a noncooperative fashion, with half-inhibition at 1.6 m*M* caffeine. After addition of 5 m*M* caffeine, the frequency of channel recordings decreased more than three-fold, although mean open time and conductance were virtually unaffected *(168).* Increasing the IP_3 concentration to 20 μ*M* overcame the inhibition, and, as with other studies *(167)* [^{3}H] IP_3 binding was unaffected.

It is unlikely that caffeine exerts its inhibitory effects by interfering with the allosteric regulation of ATP upon the receptor. ATP affects both mean open and closed times of the channel *(39),* whereas caffeine affects only the mean closed time *(168).* Caffeine activates the RyR, which is homologous to the IP_3R, at a presently unidentified site. It is likely that caffeine exerts its inhibitory effects at a site similar to the activating site on the RyR although in a different manner.

Ethanol and Other Aliphatic Alcohols

Alcohols have been reported as having diverse effects on IP_3 signaling processes *(169,170).* Measurement of Ca^{2+} mobilization revealed that methanol (2% v/v) had a negligible effect on Ca^{2+}-release, whereas ethanol (2% v/v) could both stimulate and inhibit release depending on the IP_3 concentration. Inhibition was seen with longer-chain alcohols *(169,170)* and the potency of inhibition increased with alcohol chain length, indicating that the alcohol-binding site resides within the hydrophobic region of the receptor, or at the lipid-protein interface. Potential effects of alcohols on IP_3R function are highly significant as various nonpolar drugs that are used to study the IP_3R in bilayers are dissolved in alcohols. Use of ethanol and other aliphatic alcohols as vehicles for these drugs at concentrations in the range of 1–2% (v/v) and higher could add complications to such experiments.

Polyamines

Polyamines were shown to inhibit IP3-induced Ca^{2+} release from rat cerebellar microsomes *(171)* at millimolar concentrations. Spermine, one of the most abundant naturally occurring polyamines, inhibited IP_3-induced Ca^{2+} release by increasing the IC_{50} value for the process (from 0.2 to 0.47 μ*M* in the presence of 1 m*M* spermine). In addition there was a decrease in the maximal amount of Ca^{2+} release without an effect on ligand binding. Although polyamines can bind and form complexes with IP_3, binding studies illustrated that at a concentration of 1 m*M*, spermine did not bind to IP_3, indicating that its effects were via interaction with the receptor itself *(171).* Polyamines have been shown to block caffeine-induced Ca^{2+} release through the RyR *(7).* Given the homology of the RyR with the IP_3R, a similar mechanism of action could underlie the effect in both types of channel.

Immunosuppressants: FK506 and Rapamycin

Recently, a physical association of the phosphatase, calcineurin, with the IP_3R via an immunophilin protein (FK506-binding protein [FKBP12]) has been demonstrated *(172–173).* A similar association has also been observed in skeletal muscle RyR *(45,47,174).* The com-

plexed calcineurin modulates the phosphorylation status of the IP_3R and its Ca^{2+} flux properties *(173).* Immunosuppressant drugs such as FK506 and rapamycin are able to disrupt the IP_3R-FKBP12-Calcineurin complex and dissociation has been found to increase Ca^{2+} conductance through higher phosphorylation levels of the IP_3R *(173).* In other cases FK506 inhibits Ca^{2+} release via the IP_3R *(175)* and in SH-SY5Y and A7r5 cells, it has no direct effect *(176).*

FK506 and rapamycin are widely used therapeutically for organ transplantations such as liver, kidney, heart, lung, pancreas, and intestine *(177).* However, treatment with these drugs is often associated with side effects, for example renal, hepatic, and metabolic toxicity, and hypertrophic cardiomyopathy *(177,178).* The quantities required for immunosuppression are in the nanomolar range *(179)* yet concentrations affecting Ca^{2+} signaling are at least 1000-fold higher. Given the lipophilic nature of FK506 *(180),* and the repetitive application during therapy *(177),* levels of the drug may build up in cellular membrances to significant levels, leading to altered Ca^{2+} signaling, and hence, side effects.

Heparin

Heparin is a well-known pharmacological inhibitor of the IP_3R. It inhibits IP_3R binding to the purified receptor *(32)* as well as release of Ca^{2+} from intracellular stores *(142,181)* and IP_3-induced channel activity *(28,31)* in the range of 10–100 µg/mL. Heparin has since become a standard pharmacological tool in the investigation of the IP_3R and has been used successfully in its affinity-purification from rat cerebellar membranes *(32).*

Xestospongins

Xestospongins are a group of macrocyclic bis-1-oxaquinolizidines isolated from the Australian sponge, *Xestospongia* species, and have been shown to be potent blockers of IP_3R activity *(182–183).* Xestospongin C blocks IP_3-induced Ca^{2+} release (IC_{50} = 358 nM) without affecting IP_3 binding, indicating that the inhibitory mechanism operates independently of the IP_3 effector site *(182).* Xestospongins have been valuable tools for investigating the structure and function of IP_3Rs *(182),* although a recent study suggests that they may not be as selective as originally thought *(183).* Xestospongin C was found to inhibit not only IP_3-induced Ca^{2+} flux, but also the ER Ca^{2+} pump in permeabilized A7r5 smooth-muscle cells. Nevertheless, xestospongins remain a potent class of IP_3R blockers, exhibiting a high selectivity over RyRs *(182).*

2-Aminoethoxy Diphenyl Borate (2APB)

2-Aminoethoxy diphenyl borate (2APB) is a membrane-permeant IP_3R inhibitor *(184,185).* Initially, it was found to inhibit IP_3-induced Ca^{2+} release from rat cerebellar microsomes without affecting IP_3 binding *(184).* Subsequent studies showed that 2APB also blocked capacitative Ca^{2+} entry in HEK 293 cells *(185).* One model for capacitative Ca^{2+} entry into cells, the so-called conformational-coupling model *(186–187),* suggests that the IP_3R interacts with plasma membrane Ca^{2+} entry channels *(188).* Thus, it is reasonable to conclude that 2APB blocks Ca^{2+} entry mainly by inhibition of IP_3Rs, although a direct effect on capacitative Ca^{2+} entry channels cannot be excluded *(185).*

Despite the fact that the specificity of 2APB with regards to intracellular Ca^{2+} signaling has not been completely established, it is nevertheless the first membrane-permeant modulator of the IP_3R and will provide a useful way for investigating the physiological role of the IP_3R in the cell.

Intracellular Ca^{2+} release channels (RyR and IP_3R) play an important role in a number of processes in neuronal physiology, such as neuronal development and differentiation, cell death, synaptic signaling and efficacy, memory formation, and neuronal pathology (10–17). In this article, pharmacological modulators of intracellular Ca^{2+} release channel activity have been reviewed. Rather than focusing on

endogenous modulators and ligands of RyR and IP_3R *(3,8,9,18–26,28,29),* the intent of this article has been to compile and analyze information regarding mechanisms of action and functional properties of reagents affecting intracellular Ca^{2+} release channels, primarily at the single-channel level.

RyR and IP_3R are influenced by a wide range of compounds that interact with the proteins at specific binding sites, various undefined sites, or that interfere with the ion conductivity of the channel via the level of ions or through the channel pore. Also, direct chemical modification of the channel, or the interaction of pharmacological agents with associated proteins, are important tools to assess intracellular Ca^{2+} release channel properties. The review of important modulators of the IP_3R and RyR shows that a number of pharmocological tools are available and are being developed to investigate the contribution of intracellular Ca^{2+} release channels to neuronal physiology. Equally important, this article also shows that a number of drugs and classes of compounds that are being used extensively to investigate other physiological processes have complex effects on intracellular Ca^{2+} release channels. Therefore the information provided in this article will help not only to understand the function of intracellular Ca^{2+} release channels in neurons and to promote the investigation of such processes. It will also be useful to identify new tools for functional analysis and to help evaluate the properties of modulators of IP_3R and RyR entering the field of molecular neurobiology.

Acknowledgments

We thank B. Ehrlich, M. Koulen, S. Koulen, and B. Sakowska for generous support and encouragement.

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