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COMMENTARY

VOLTAGE-INDEPENDENT CALCIUM CHANNELS

REGULATION BY RECEPTORS AND INTRACELLULAR CALCIUM STORES

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Calcium plays an essential role in a number of diverse physiological processes including nerve conduction, cell adhesion and migration, and cell growth and differentiation. Changes in cellular calcium homeostasis are regulated, in part, through the opening and closing of calcium permeable channels located in the plasma membrane. Considerable evidence has accumulated describing both VOCCs§, which are regulated by membrane potential, and ligand-gated cation channels in which receptor and channel are part of the same macromolecular protein complex. VOCCs and ligand-gated cation channels are expressed primarily in excitable cells, such as neurons and muscle cells, and their role in physiology and pathophysiology has been well established [1]. More recently, however, calcium influx has been described following receptor activation, which can occur through VICCs [2, 3]. Voltage-independent calcium influx has been identified by electrophysiological and biochemical techniques and has been described in nonexcitable cells, such as epithelial and humoral cells, and stimulated by many ligands including neurotransmitters, hormones, cytokines, and antigens. The molecular mechanism of VICC regulation is presently controversial since receptor-dependent and receptor-independent calcium influxes have been described. It is not clear if receptor-coupled VICCs play a role in the refilling of IP_3-sensitive intracellular calcium pools or if the refilling process occurs through ion exchange or transport. Evidence indicates that VICCs constitute a large family of proteins, with different family members being expressed in excitable cells and nonexcitable cells, each with their own regulatory mechanisms and biological functions. VICCs have been implicated in the regulation of cell growth and differentiation, and possibly in malignant transformation. This commentary will focus on evidence from two experimental approaches, electrophysiology and fluorescence spectroscopy, which demonstrates increasing diversity in the properties of receptor-regulated calcium influx.

Three main mechanisms of voltage-independent calcium influx are now proposed (Fig. 1): (a) ROCC activation that is independent from regulation by a diffusible second messenger and tightly coupled to the receptor; (b) DOCC activation that provides a source of calcium for refilling of intracellular calcium stores and is regulated by the concentration of calcium within the store; and (c) SMOCC activation that is regulated by calcium itself, IP_3, IP_4, or other second messengers released following receptor activation, but that is independent of the filling state of intracellular calcium stores. A brief overview of electrophysiological and biochemical methods used to characterize VICCs, as well as evidence for each of these three models, is summarized below.

Electrophysiological measurements of VICCs

Electrophysiological studies have provided detailed information about voltage-independent calcium influx. Voltage clamp, in which membrane potential is held constant while transmembrane ion movement or current is measured, was originally used to characterize Na^+ and K^+ channels in the squid giant axon [4–7] and, more recently, to measure calcium influx in single cells. The improved "patch" voltage clamp technique allows measurement of single channels [8]. The recording electrode is a glass micro-pipet that can be used in three configurations, allowing for slightly different recording characteristics. (1) The whole cell configuration of patch clamp, in which the electrode penetrates the plasma membrane, has the advantage that the intracellular solution can be controlled easily. In addition, the measurement of channels with small conductances are more easily detected

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§ Abbreviations: VOCC, voltage-operated calcium channel; VICC, voltage-independent calcium channel; ROCC, receptor-operated calcium channel; DOCC, depletion-operated calcium current; and SMOCC, second messenger-operated calcium channel.

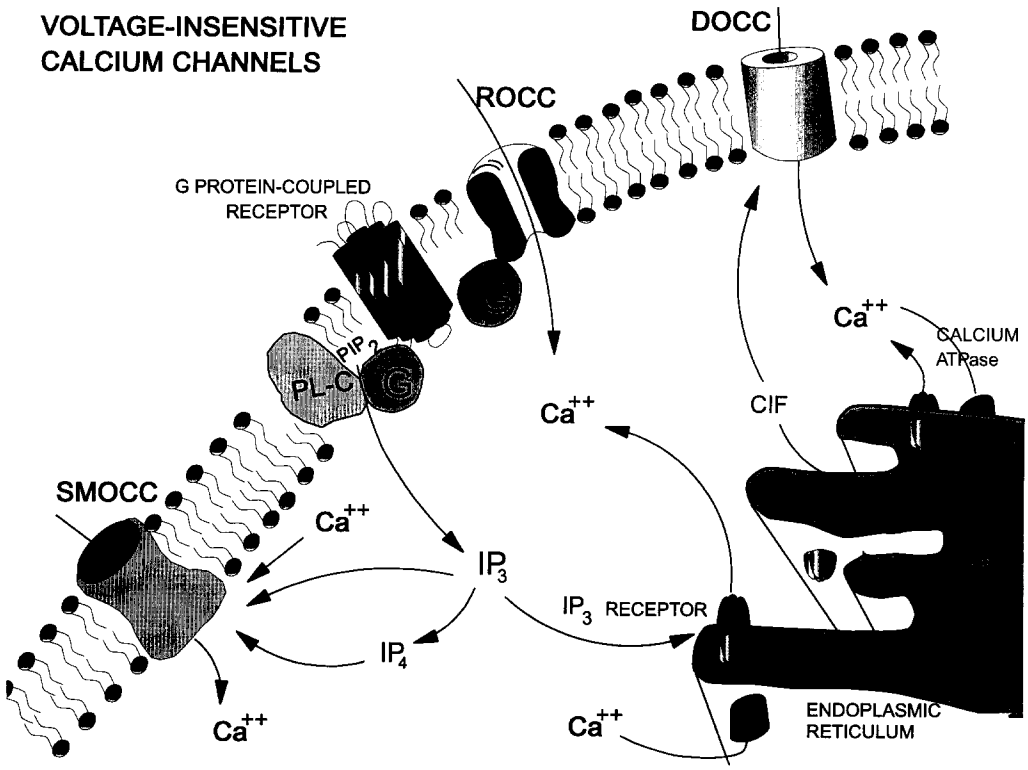

Fig. 1. Calcium influx across the plasma membrane through voltage-independent calcium channels (VICCs). G protein-coupled receptors, characterized by their seven transmembrane domains, can initiate calcium influx through three VICC types: (1) receptor-operated calcium channels (ROCCs) that are independent of second messenger or calcium regulation and may be regulated through G proteins, (2) depletion-operated calcium channels (DOCCs) that are presumably regulated by a cytoplasmic calcium influx factor (CIF) released following depletion of calcium from the endoplasmic reticulum. Calcium ATPases pump cytoplasmic calcium back into the lumen of the endoplasmic reticulum and, (3) second messenger-operated calcium channels (SMOCCs) that are regulated by IP_3, IP_4, and intracellular calcium generated following receptor stimulation.

since the current measured is the sum of the amplitudes of the currents from the population of channels expressed in the entire cell. The disadvantage of whole cell patch clamp is the tendency for currents to diminish after short periods of time (run-down), as well as the dialysis of cytoplasmic constituents with the pipet solution leading to the loss of regulatory second messengers. As a remedy to the problems of whole cell voltage clamp, nystatin perforated-patch recording was developed. Application of nystatin, an antibiotic, generates 0.8-nm diameter channels in the plasma membrane under the patch, which are permeable to monovalent cations, and to a lesser extent Cl^-, but not to divalent cations and larger molecules [9]. The disadvantages of this technique include loss of control over the composition of the intracellular solution and the technical difficulty of its execution. It appears, however, to be ideally suited for the study of VICCs, some of which appear to be regulated through intracellular mechanisms. (2) Single channel openings can be recorded from intact cells in the cell-attached configuration in which the measuring electrode is placed against the surface of the cell and allowed to create a seal with high electrical resistance. The cell-attached configuration can provide information about the second messenger dependency of VICC activation. For example, second messenger-independent ROCC activation can only occur when agonist, receptor, and channel are closely associated within the area of membrane trapped under the microelectrode, and no response results from bathing the membrane with agonist outside the pipet. (3) Although more technically demanding, excised membrane patches can provide access to either the cytoplasmic surface (inside-out configuration) or to the exterior of the plasma membrane (outside-out configuration). The patches of membrane that are torn from the cell are essentially free of cytoplasmic constituents. Channels are presumably activated by membrane-associated constituents or reagents applied to the cell in the medium. One must consider, however, that cell-free patches may include cytoplasmic components that associate tightly with the plasma membrane. These electrophysiological techniques have allowed direct measurement of, and proven essential for, full characterization of VICCs.

Fluorescence spectroscopy

Early attempts at measuring calcium in single cells

used a variety of techniques including $^{45}Ca^{2+}$ uptake and efflux, acquorin luminescence, bis-azo absorbance dyes, and ion-specific electrodes that were difficult to use and restricted to well-equipped laboratories [for a review, see Ref. 10]. The development of ratiometric fluorescent dyes, such as Quin-2, Fura-2, and Indo-1, that change their fluorescent properties after binding to free calcium, has made the measurement of cytosolic free calcium in single cells considerably more accessible [11]. These calcium-chelating compounds have affinities for calcium, which allow reliable quantification of intracellular free calcium concentrations over the physiological agonist-induced range, from a resting level of approximately 10 nM to maximum levels of several micromolar. The most popular dye, Fura-2, is excited at two wavelengths, 340 and 380 nm, and the emission of the two wavelengths is monitored at 510 nm. Increasing free cytoplasmic calcium concentrations results in an increase in the 340 nm/380 nm ratio value. The advantage of ratiometric fluorescent dyes is that variabilities due to dye concentration are cancelled out during the ratio calculation. Photometry or video camera imaging of fluorescent dye-loaded cells has established two paths for calcium to accumulate in the cytoplasm, either through release from intracellular stores or influx through calcium permeable ion channels. Temporal measurements of receptor-stimulated cytoplasmic free calcium have been typically characterized by an initial spike, which then declines over seconds to several minutes to a plateau phase that decays more slowly or, in some cases, can persist as long as agonist is present. It is well accepted that the initial spike is the result of receptor-mediated production of the second messenger IP_3, which releases calcium from cytoplasmic stores [12]. In most cells, removal of external calcium causes a decay of the plateau phase to baseline values, indicating that its maintenance is dependent on calcium influx. Further verification of calcium influx has been shown through substitution of manganese for calcium in the external medium. Agonist-stimulated manganese influx appears to follow similar calcium entry pathways resulting in quench of the fluorescent signal in Fura-2-loaded cells [13]. Interpretation of manganese-quench experiments must be done with caution, however, because of the possible multiple mechanisms of manganese influx [14]. Although fluorescent spectroscopy does not directly measure individual calcium or cation channel activity, a significant understanding of receptor-operated calcium entry has been derived from these types of experiments.

Receptor-operated calcium channel (ROCC)

Receptor-stimulated calcium influx through voltage-insensitive ROCC type channels occurs without the requirement of cytoplasmic messengers. The first direct measurement of ROCC activation was demonstrated through electrophysiological recordings of ATP-stimulated rabbit ear smooth muscle cells [15]. This study suggested that diffusible second messengers and intracellular calcium did not play a role in the regulation of this ROCC, since ATP-stimulated channel activation was observed in outside-out excised membrane patches, a condition under which it is assumed that diffusible second messengers are diluted in the electrode solution and become unavailable. Moreover, no channel activity was observed when the cell was bathed in ATP in the cell-attached configuration. This channel had a 3:1 selectivity of Ca^{2+} over Na^+, a conductance of about 5–10 pS, was insensitive to membrane depolarization or inhibitors of VOCCs, and was equally permeable to Ba^{2+} and Mg^{2+}. These characteristics were clearly different from classical voltage-operated calcium channels, which have a 1000-fold selectivity for Ca^{2+} over Na^+, open on membrane depolarization, are blocked by selective inhibitors such as dihydropyridines, and are impermeable to Mg^{2+}. Inositol phosphate and intracellular calcium-independent ROCC activation was shown in human platelets using Fura-2 and stopped-flow fluorimetry [16]. ROCC activation preceded the release of calcium from IP_3-sensitive stores, removing the possibility of store depletion or the IP_3-stimulated calcium spike as mediators in the channel regulation. This group further demonstrated, using the cell-attached patch clamp technique, that channel activation occurred only when ADP was included in the electrode solution, further supporting its independence from a diffusible second messenger [17]. Similar studies using the cell-attached configuration or whole cell configuration in rat parotid acinar cells [18], murine thymocytes [19], and mouse lacrimal cells [20] support the existence of second messenger-independent ROCCs. Muscarinic receptor stimulation of IP_3- and calcium-independent ROCCs was also observed in Fura-2-loaded fibroblasts [21].

ROCC activation is presumed to be second messenger-independent based primarily on electrophysiological data described above; therefore, regulation of ROCCs by receptors must occur through a membrane-bound constituent, such as a G protein, or through direct receptor–channel interaction. Evidence for G protein-mediated ROCC regulation is supported through the use of non-hydrolyzable guanine nucleotide analogs, which modulate all G protein subtypes, and pertussis toxin, which blocks both G_i and G_o. Definitive proof of which G protein subtype mediates channel regulation will require either reconstitution of purified receptor, appropriate G protein, and channel (when they become available) into artificial membrane vesicles, or will require the ectopic expression of the channel cDNA into mammalian host cells once the channels are cloned. It is possible that G protein-coupled receptors use more than the third cytoplasmic loop (the G protein binding site) for calcium channel regulation or that regulation is G protein independent. These possibilities were addressed in A9 fibroblasts transfected with and stably expressing muscarinic acetylcholine receptors [21]. Muscarinic m3 receptor-stimulated calcium influx was shown to be independent of inositol phosphate generation and intracellular calcium release and, therefore, independent of the filling state of the IP_3-sensitive intracellular calcium pool. Inhibition of IP_3-stimulated intracellular calcium release with phorbol ester allowed direct visualization of ROCC-mediated

Table 1. Characteristics and proposed nomenclature of voltage-independent calcium channels

Type of channel	Regulation	Conductance	Permeation
ROCC-1 (receptor-operated calcium channels)	Receptor G-protein (?)	3–10 pS	Ca^{2+}, Ba^{2+}, $Mn^{2+} > Na^{+}$
ROCC-2 (non-specific cation channels)	Receptors Internal Ca^{2+} Stretch G-protein	20–50 pS	Ba^{2+}, Ca^{2+}, Na^{+}
DOCC (depletion-operated calcium currents)	CIF IP_3/IP_4 cGMP G-protein (?) Cytochrome P450 (?) Ionophore Thapsigargin Ca^{2+} chelators	<1 pS	$Ca^{2+} \gg Ba^{2+}$, Sr^{2+}, Mn^{2+}
SMOCC (second messenger-operated calcium channels)	IP_3/IP_4 Ca^{2+}	4–25 pS	Ba^{2+}, Ca^{2+}, $Mn^{2+} > K^{+}$

calcium influx. Possible G protein-mediated regulation was addressed through exchange of the third cytoplasmic loop of the m2 and m3 receptor using molecular techniques. The wild-type m2 receptor had no effect on calcium mobilization, and the wild-type m3 receptor stimulated calcium influx and mobilization from intracellular calcium stores. Replacement of the m3 third cyoplasmic loop for the m2 loop conferred on the m2 receptor the ability to stimulate IP_3 and cytoplasmic calcium release. The chimeric m3 receptor containing the m2 loop retained its ability to stimulate calcium influx through a ROCC. The conclusion of this study was that the m3 third cytoplasmic loop was not involved in receptor–ROCC interaction but was essential for IP_3 generation and subsequent intracellular calcium release.

Non-specific cationic channels offer an alternate route for calcium influx. These channels have been identified in a number of cell lines including human neutrophils [22], frog lens epithelia [23], human vascular endothelial cells [24], rat mast cells [25], and rat liver cells [26], under various forms of regulation including receptors, internal calcium, and mechanical stretch. They typically have conductances of approximately 20–50 pS and are calcium permeable but not calcium selective. A subset of these channels are receptor-operated and second messenger-independent and, therefore, should be included in the ROCC family of channels. Others are second messenger- or calcium-dependent and should be included with the second messenger-regulated channels described below [22]. General characteristics of calcium selective and non-specific cation channels, along with proposed nomenclature, are listed in Table 1. Non-specific cation channels with conductances of 50 pS were shown in rat mast cells that were IP_3- and intracellular calcium-independent, but responded to the application of GTP-γ-S, suggesting G protein regulation [27]. Therefore, the ROCC channels may represent a diverse family of proteins displaying variations in ion selectivity, reversal potential, conductances, and regulation depending on the cell in which it is expressed. Most ROCCs described to date have in common a conductance of approximately 3–5 pS, an insensitivity to blockers of VOCCs, an impermeability to heavy metals such as Cd^{2+} or La^{3+}, but permeability to Mn^{2+}, Ba^{2+} and Ca^{2+}, are second messenger independent, and are not regulated by calcium itself.

Several compounds have been described that either block or stimulate calcium entry through ROCCs. The carboxyamido-triazole, CAI, has been shown to block ROCCs and ROCC-dependent signaling, but is somewhat non-selective in that it also blocks VOCCs and ionophore-mediated calcium influx [28–30]. An interesting feature of this compound is its antimetastatic and antiproliferative properties [31]. SKF96365 [32] and other antimycotic imidazoles, including econozole and miconazole, have shown promise as ROCC inhibitors, but are far from selective. Maitotoxin, a potent marine toxin isolated from the dinoflagellate *Gambierdiscus toxicus*, activates calcium entry through ROCCs, and possibly other channels, and is not ionophoretic [33].

Depletion-operated calcium current (DOCC)

The first suggestion that empty IP_3-sensitive intracellular calcium pools recruit plasma membrane calcium influx for refilling of the calcium pools was made by Michell in 1975 [34]. Since then, two hypotheses have predominated to explain the calcium pool refilling process. The capacitative model, first described by Putney in 1986 [35], currently suggests that receptor-generated IP_3 is responsible for store depletion, and that a cytoplasmic signal or messenger is sent from the depleted store to the plasma membrane to activate DOCCs. The second hypothesis provides two roles for IP_3: first, the depletion of intracellular calcium pools, and second, the direct opening of plasma membrane calcium channels to provide the calcium necessary to refill the pools. Evidence also suggests that IP_3 is phosphorylated to IP_4, which then may stimulate calcium channel activation. Whether the calcium influx described by the second hypothesis plays a role in pool refilling has not been firmly established, and this class

of second messenger-operated channels will be discussed further below.

The capacitative model is supported by the discovery of agents that deplete intracellular calcium pools without the requirement of receptor stimulation or inositol phosphate release. These agents act by either inhibiting microsomal Ca^{2+}-ATPases responsible for moving calcium from the cytoplasm to the intracellular calcium pools or through ionophoretic release. Three molecularly distinct Ca^{2+}-ATPase inhibitors have been identified, thapsigargin [36], 2,5-di-(*tert*-butyl)-1,4-hydroquinone (BHQ) [37], and cyclopiazonic acid (CPA) [38], and along with ionophores, such as A23187 or ionomycin, provide useful pharmacological tools to deplete intracellular calcium pools. Using this approach, ample evidence has accumulated using fluorescence spectroscopy to support the capacitative refilling model in a variety of cell types [39–45]. The first electrophysiological measurement of DOCCs in mast cells (named I_{CRAC} by these authors for calcium release-activated calcium current) revealed a calcium current that was highly selective for calcium over Ba^{2+}, Sr^{2+}, and Mn^{2+} [46]. DOCCs were activated by IP_3, but also by ionophore, and a calcium chelator. Moreover, thapsigargin-activated DOCCs have been reported, suggesting that calcium influx is not mediated by IP_3 directly [47]. Since these initial electrophysiological characterizations, DOCCs have been measured in a variety of cell types but best characterized in mast cells [48] and human T cells [47, 49]. Single channel openings of DOCCs have not been measured, but have been estimated at below 1 pS [48] and by noise analysis at 20 fS [47]. It may be that DOCC conductance is below the resolution of available techniques or that it is not an ion channel.

Excitable cells were thought to express only VOCCs and not VICCs [3, 50]. Recent work has demonstrated DOCCs in excitable cell types including muscarinic receptor-stimulated DOCC activation in N1E-115 neuroblastoma cells [51] and PC12 cells [52]. The DOCC in NIE-115 cells was the first fully characterized in an excitable cell using the nystatin patch clamp technique and was shown to be stimulated by either thapsigargin or muscarinic receptor activation. Like DOCCs in nonexcitable cells, this current was shown to be calcium selective, voltage insensitive, and inhibited by Ba^{2+} and Mn^{2+}.

Several mechanisms have been suggested for DOCC regulation including the release of a diffusible cytoplasmic second messenger from the endoplasmic reticulum following calcium pool depletion, which would stimulate DOCC activation at the plasma membrane. Two groups have provided preliminary evidence that such a molecule exists. Randriamampita and Tsien [53] described a calcium influx factor (CIF) in Jurkat T-lymphocyte cells, which has a small molecular weight, contains a phosphate molecule, is membrane permeable, and stimulates pool refilling. Parekh and coworkers [54] described a soluble phosphate containing messenger involved in calcium influx in *Xenopus* oocytes through the technique of "cramming" an excised membrane patch back into the cell to restore calcium channel activation. Other modulators have been linked to DOCC activation. Cyclic GMP was shown to modulate DOCCs in pancreatic acinar cells [55] and, with its single phosphate, may be similar to the CIF molecule, although CIF was shown not to be cGMP [53]. A GTP-dependent protein may also be involved in DOCC regulation. Through the use of guanine nucleotide analogs, two groups have shown that a GTP hydrolysis step, and not simply GTP binding, is necessary for DOCC activation [56, 57]. The nature of the GTP-hydrolyzing protein is currently not known, but it does not appear to belong to the family of large hetrotrimeric G proteins responsible for coupling effectors to enzymes or proteins to receptors. Recently, tyrosine kinase inhibition was shown to attenuate calcium influx mediated by bradykinin and thapsigargin, providing a role for tyrosine kinases in DOCC regulation [58]. Cytochrome P450 enzyme activity has also been implicated in DOCC regulation [59], but this hypothesis remains controversial [2]. It is also possible that in some cells calcium pools are not refilled through a highly regulated process, but are "continually fed" by a leak of calcium across the plasma membrane [60, 61].

Second messenger-operated calcium channel (SMOCC)

Calcium-mobilizing receptors stimulate the release of IP_3, which then binds to its own ligand-gated ion channel receptor on the endoplasmic reticulum or calciosome [62] to trigger the release of calcium from cytoplasmic pools [for a review, see Ref. 12]. Both IP_3 and IP_4 have been implicated as regulators of calcium influx [63–66]. IP_3 may work alone or in conjunction with IP_4 to promote calcium entry, depending on the cell type. IP_3-sensitive and IP_4-insensitive calcium influx has been suggested previously [67, 68], but these studies could not rule out the potential conversion of IP_3 to IP_4 since IP_3 kinase appears to be widely distributed. Conflicting data in mouse lacrimal cells suggest that both IP_3 and IP_4 are required to maintain the calcium influx following muscarinic receptor stimulation [69]. IP_4-independent stimulation of calcium influx was definitively demonstrated in lacrimal acinar cells using a non-phosphorylatable IP_3 [70]. More recently, evidence demonstrating direct activation of a 2.5-pS cation channel by Ca^{2+} and IP_4, but not IP_3, was shown in endothelial cell membrane patches, providing convincing evidence for a role for IP_4 in calcium influx regulation in these cells [71].

Are SMOCCs involved in the refilling of intracellular pools? Activation of SMOCCs by Ca^{2+}, IP_3, or its metabolites provides a logical connection between activated receptor and the refilling process. Under this arrangement, intracellular calcium release and the refilling process could proceed economically, regulated by the same messenger. However, characteristics of SMOCCs shown to be activated by inositol phosphates or calcium do not fit the profile of the low conductance DOCCs described above. SMOCCs have conductances that range between 4 and 25 pS, are permeable to manganese, and are insensitive to thapsigargin (see Table 1). Furthermore, thapsigargin activates calcium influx without elevating IP_4 or IP_3 levels. It remains to be

determined if, in all cells, DOCCs stimulated through artificial store depletion (e.g. thapsigargin) are identical to those recruited following receptor activation, as they are in carbachol-stimulated N1E-115 cells [51] and mitogen-stimulated T lymphocytes [47]. It is also not clear what role SMOCCs and ROCCs play in the refilling process. Conclusions about the role of SMOCCs will require more examples derived from a variety of cell lines.

Physiologic role

The role of DOCCs in refilling IP_3-sensitive pools is now firmly established. Recent evidence has shown a close relationship between DOCCs and calcium pools in the regulation of calcium oscillations, suggesting that its role in regulating calcium homeostasis is far from simple [72]. The identification and characterization of ROCCs and SMOCCs have occurred only recently; therefore, their physiologic role is still somewhat speculative. Although store depletion does not activate SMOCCs and ROCCs, calcium influx through these channels must contribute to the store refilling process. These channels are also likely to play an important part in calcium influx-dependent biological events, such as cell growth, differentiation, migration, and secretion [73]. These events are regulated by calcium influx-dependent signal transduction proteins such as phospholipases, kinases, and phosphatases. A recent study has linked muscarinic receptor-stimulated calcium influx through ROCCs to the reversal of CHO cells from a tumorigenic to a non-tumorigenic phenotype [74]. Considerable progress is being made in sorting out the role of a DOCC channel in invertebrate visual phototransduction [75, 76]. Light-induced IP_3 generation resulted in the activation of a DOCC that is decreased or missing in a *Drosophila* trp gene mutant. This discovery made lead to the cloning of members of the VICC family of channels. Future progress should reveal if each VICC type has a discrete biological function, or if their roles overlap.

Evidence reviewed above clearly reveals a family of VICCs, each with their unique characteristics and mode of regulation. Simultaneous measurement of DOCC and ROCC [25, 60] or ROCC and SMOCC [27] in the same cell removes any doubt that separate proteins represent each channel type. It is likely that diversity will exist in each of these channel families and that new lines of definition will be drawn as this field progresses and the channels are cloned. Future challenges include isolation of VICC channels, cloning and mapping their distribution, and characterizing the diversity and physiologic function of the various family members.

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