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Ca^{2+} Channels on the Move[†]

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ABSTRACT: The versatility of Ca^{2+} as an intracellular messenger derives largely from the spatial organization of cytosolic Ca^{2+} signals, most of which are generated by regulated openings of Ca^{2+}-permeable channels. Most Ca^{2+} channels are expressed in the plasma membrane (PM). Others, including the almost ubiquitous inositol 1,4,5-trisphosphate receptors (IP$_3$R) and their relatives, the ryanodine receptors (RyR), are predominantly expressed in membranes of the sarcoplasmic or endoplasmic reticulum (ER). Targeting of these channels to appropriate destinations underpins their ability to generate spatially organized Ca^{2+} signals. All Ca^{2+} channels begin life in the cytosol, and the vast majority are then functionally assembled in the ER, where they may either remain or be dispatched to other membranes. Here, by means of selective examples, we review two issues related to this trafficking of Ca^{2+} channels via the ER. How do cells avoid wayward activity of Ca^{2+} channels in transit as they pass from the ER via other membranes to their final destination? How and why do some cells express small numbers of the archetypal intracellular Ca^{2+} channels, IP$_3$R and RyR, in the PM?

LOCAL AND GLOBAL CA^{2+} SIGNALS

The plasma membrane (PM)[1] and the membranes of many intracellular organelles separate the cytosol, with its low resting free Ca^{2+} concentration of ~100 nM, from environments with very different free Ca^{2+} concentrations and electrical potentials. The resulting steep Ca^{2+} gradients are poised to allow Ca^{2+} to flow rapidly down its electrochemical gradient whenever Ca^{2+}-permeable channels within these membranes are open. For the PM and most organelles, notably the endoplasmic reticulum (ER) and organelles derived from it, the gradients are directed toward the cytosol. Regulated opening of Ca^{2+} channels within these membranes is the means by which most extracellular and intracellular signals evoke the increases in cytosolic Ca^{2+} concentration that regulate almost every aspect of cellular activity (*1, 2*). For other membranes, the inner membrane of mitochondria and perhaps of chloroplasts (*3*), the Ca^{2+} gradients are directed away from the cytosol so that channels within these membranes, MiCa within the inner mitochondrial membrane, for example (*4*), mediate uptake of Ca^{2+} from the cytosol. Each of these membranes is also home to proteins that transport Ca^{2+} in the opposite direction, against its electrochemical gradient, the Ca^{2+}-ATPases of the PM (PMCA), ER (SERCA), and the Golgi and secretory vesicles (SPCA) (*5−7*), for example. The competing activities of Ca^{2+} channels and the pumps and exchangers that move Ca^{2+} up its electrochemical gradient ultimately determine the cytosolic Ca^{2+} concentration, but it is the Ca^{2+}-permeable channels that mediate the most rapid Ca^{2+} exchanges and which are most commonly acutely regulated by signaling pathways.

Each of these Ca^{2+}-permeable channels allows Ca^{2+} to pass through a central pore traversing a biological membrane. They differ, however, in whether under physiological conditions they effectively allow only Ca^{2+} to pass [e.g., voltage-gated Ca^{2+} channels (Ca$_v$) and the Orai proteins that mediate store-operated Ca^{2+} entry] or also allow other cations to pass [e.g., IP$_3$ receptors (IP$_3$R), ryanodine receptors (RyR), and nicotinic acetylcholine receptors]. The difference, defined by the structure of the selectivity filter (*8*), is significant because it determines whether the channels, in addition to mediating Ca^{2+} fluxes, can also regulate membrane potential (most important at the PM) and conduct the counterions required to allow electrogenic movement of Ca^{2+} (*9*).

Because the cytosol of all cells contains high concentrations of Ca^{2+} buffers, Ca^{2+} diffuses more slowly in cytosol than in free solution (*10, 11*). An important consequence is that as Ca^{2+} flows rapidly through an open Ca^{2+} channel, it creates a local cloud with a high cytosolic Ca^{2+} concentration: each active channel creates its own local Ca^{2+} signal (*12*). These spatially organized Ca^{2+} signals are important because different Ca^{2+}-binding proteins selectively associate with different Ca^{2+} channels, so that Ca^{2+} passing through one channel may regulate different events to Ca^{2+} passing through another (*2*). Store-operated Ca^{2+} entry (SOCE) (*13*), for example, has been reported to regulate selectively the Ca^{2+}-sensitive adenylyl cyclases (*14*), nitric oxide synthase (*15*), and gene expression in rat basophilic leukemia cells (*16*). Cardiac IP$_3$R associate with a Ca^{2+}-regulated protein kinase that also regulates IP$_3$R activity [Ca^{2+}-calmodulin-dependent

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[1]Abbreviations: AKAP, A-kinase-anchoring protein; BCR, B-cell receptor; CaMKII, Ca^{2+}-calmodulin-dependent protein kinase II; CaR, Ca^{2+}-sensing receptor; Ca$_v$, voltage-gated Ca^{2+} (channel); 4CmC, 4-chloro-*m*-cresol; CNG, cyclic nucleotide-gated (channel); ER, endoplasmic reticulum; ERAD, ER-associated degradation; γ, single-channel conductance; IP$_3$, inositol 1,4,5-trisphosphate; IP$_3$R, IP$_3$ receptor(s); PIP$_2$, phosphatidylinositol 4,5-bisphosphate; PKA, cyclic AMP-dependent protein kinase; PM, plasma membrane; PMCA, plasma membrane Ca^{2+}-ATPase; RyR, ryanodine receptor(s); SERCA, SR/ER Ca^{2+}-ATPase; SPCA, secretory pathway Ca^{2+}-ATPase; SOCE, store-operated Ca^{2+} entry; SR, sarcoplasmic reticulum; SRP, signal recognition particle; STIM, stromal interaction protein; TGN, trans-Golgi network; TRP, transient receptor potential.

protein kinase IIδ (CaMKIIδ)], so that release of Ca^{2+} via IP_3R may selectively activate an enzyme that then feeds back to inhibit IP_3R activity (*17*). Ca^{2+} entry via Ca_v channels (Ca_v1 and Ca_v2) selectively regulates the activity of the channel itself (*18*). The latter highlights another key feature of many Ca^{2+} channels, namely their regulation by cytosolic Ca^{2+}. This provides feedback regulation of Ca^{2+} signaling, and it allows Ca^{2+} channels to evoke regenerative Ca^{2+} signals (*19*). The latter are important because they underpin the versatility of Ca^{2+} as an intracellular messenger, permitting it to function either locally or globally (*2*). It follows from this discussion that the versatility of Ca^{2+} as a ubiquitous intracellular messenger derives in large part from putting Ca^{2+}-permeable channels into the right place; only then can they deliver spatially organized Ca^{2+} signals.

PUTTING CA^{2+} CHANNELS IN THE RIGHT PLACE

A few proteins, mostly those related to transcription and translation, but also components of a H^+ channel (ATP synthase), are encoded by DNA within mitochondria and chloroplasts. These proteins are synthesized within these organelles and so reach their final destination without traversing the cytoplasm. All other proteins are encoded by nuclear DNA. These proteins are synthesized in the cytosol from where they are dispatched to other destinations. Targeting of proteins has attracted the most attention (*20*, *21*), but many proteins are guided toward their final destination before translation begins by selective targeting of mRNA (*22*).

After transcription and mRNA processing within the nucleus, mature mRNA is exported via nuclear pores to the cytosol. Even this step may contribute to mRNA targeting. Synthesis of mRNA encoding some subunits of nicotinic acetylcholine receptors, for example, is restricted to the nuclei lying immediately beneath the neuromuscular junction of multinucleate muscle fibers (*23*). More generally, transport of mRNA within the cytosol, mediated by binding of proteins to sequences within the 3′-untranslated region, probably plays the major role in selective trafficking of mRNA (*22*, *24*, *25*). Such transport can direct mRNA to associate with the membranes of specific organelles. The mRNAs for approximately half of all mitochondrial proteins, for example, including the mRNA for another subunit of the ATP synthase, are directed to the outer mitochondrial membrane (*25*). Other mRNAs are selectively addressed to nuclear membranes, the ER (*26*), and even to specific subdomains of the ER (*27*). Transport of mRNA also occurs over much larger distances, allowing its selective targeting to specific cytosolic destinations such as dendrites (*24*), immature axons (*28*), and polarized regions of developing embryos (*22*). The mRNAs for IP_3R1 (*29*), glutamate receptors, and a major neuronal Ca^{2+} sensor (CaMKIIα) are transported to dendrites by microtubules within "mRNA granules" that include ribosomes and additional proteins. Within these "ready-to-translate" granules (*27*), translation is probably repressed until the complex reaches its destination, where protein synthesis can proceed close to the final destination (*24*, *28*). The advantages of selectively transporting mRNA, rather than protein, have been much discussed (*22*, *30*, *31*). Clearly, mRNA transport can complement the additional targeting provided by proteins, but other advantages include cost (cheaper to move mRNA than all the proteins to which it will give rise), restricting production of potentially toxic proteins to their target site, speeding the rate at which protein synthesis can adapt to local needs (e.g., within postsynaptic
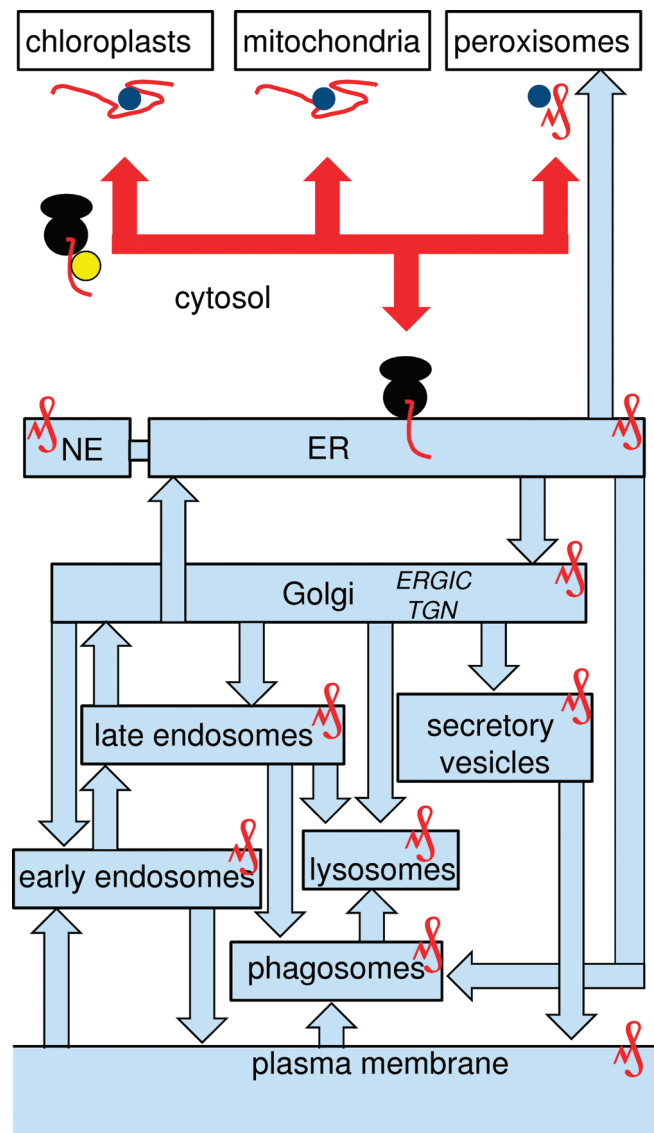

FIGURE 1: Targeting channels to different membranes from the cytosol. Protein synthesis begins in the cytosol before targeting to specific organelles (red lines). This can occur post-translationally as unfolded proteins (mitochondria and chloroplasts) or fully folded proteins (peroxisomes), each protein protected by chaperones (blue circles). Most ER targeting occurs cotranslationally, mediated by SRP (yellow) binding to a signal sequence. Once inserted into the ER membrane, proteins assume the topology that they will retain through all subsequent trafficking steps. Some proteins may pass to the nuclear envelope (NE) or be exported directly from the ER to peroxisomes, but all other proteins pass via the ER−Golgi intermediate compartment (ERGIC) to the Golgi before being sorted to various destinations from the trans-Golgi network (TGN). Routes to and from the PM are shown.

structures) (*24*), facilitating cotranslational assembly of multi-subunit proteins, and establishing protein gradients, notably during development. For Ca^{2+} channels destined to function in membranes derived from the ER, mRNA targeting may also provide a means of ensuring that functional channels are assembled near their final destination and so spend less time en route via organelles within which they may function inappropriately.

All proteins encoded by nuclear DNA, even those encoded by mRNA that associates with specific membranes, begin life in the cytoplasm where protein synthesis is initiated. Integral membrane proteins, which comprise ~30% of all proteins, are then

directed to specific intracellular membranes. These decisions are dictated by short signal sequences, address labels, within the nascent protein chain (*32*). The identities of these sequences are generally better understood for proteins destined for the lumen of organelles than for those targeted to membranes (*20, 21*). The sequences, whether N-terminal (which are often cleaved later) or internal, are recognized by cytosolic proteins, which then interact with receptors in the target membrane. There are rather few immediate destinations for integral membrane proteins: the membranes of mitochondria or, in plants, of chloroplasts; peroxisomes; and the ER (Figure 1). With the exception of peroxisomes, where membrane proteins can be fully synthesized and assembled before being dispatched from the cytosol to the peroxisome import machinery (*33*), all membrane proteins are imported into membranes from the cytosol in a state that is at least partially unfolded. Mitochondrial and chloroplast proteins are fully translated, held in their unfolded state by association with cytosolic chaperones, and then dispatched to the appropriate membrane, where they fold, integrate into the lipid bilayer, and assemble with other proteins. For both organelles, signal sequences guide the protein through the outer and, where appropriate, inner membrane via protein translocases ("translocons"). The transmembrane regions of the folded protein can then either escape laterally from the translocase into the lipid bilayer or reinsert into the outer or inner membrane after release into the intermembrane space or matrix, respectively (*34, 35*). The key point, in the context of this review, is that membrane proteins destined for mitochondria, chloroplasts, or peroxisomes are functionally assembled at their final destination: they need not visit other organelles once they become functional. The situation is very different for the many membranes that acquire their proteins from the ER.

TARGETING CA²⁺ CHANNELS TO THE ER AND BEYOND

Most ER proteins are cotranslationally targeted. The signal sequence is recognized as it emerges from the ribosome by the signal recognition particle (SRP), translation is arrested, and it resumes only after SRP has conveyed the ribosome-nascent chain complex to the ER and engaged the Sec61 translocon. The growing protein is then threaded through the translocon. A lateral gate within the translocon appears to allow proteins to become exposed to the lipid bilayer as they pass through the translocon, so that hydrophobic transmembrane helices can diffuse, alone or in pairs, through the gate and into the ER membrane (*36, 37*). There, they can assemble with other helices or protein subunits. Some ER-targeted proteins, those with C-terminal signal sequences, follow the same route but after translation is complete (*38*). An essential point is that the topology of every membrane protein—dictated by whether it has a cleavable signal sequence, the number of transmembrane helices, and their preference for aligning with the more positively charged end in the cytosol—is determined once and for all by the initial integration events (*39*). This applies to all membrane proteins, but it deserves particular emphasis for those targeted to the ER, from where many proteins will progress to other organelles. Because this later trafficking involves moving the protein within its membrane, the topology of the protein is invariably preserved: the surface of the protein that was cytosolic when it first integrated into the ER membrane remains so wherever the protein goes (*39*) (Figure 1).

A safeguard against errors in the long sequence from DNA to assembled protein is provided by the proofreading abilities of the ER. The ER-associated degradation (ERAD) pathway ensures that only properly folded proteins are allowed to remain within the ER or progress onward. Misfolded proteins are recognized by chaperones, exported back to the cytosol, probably through the Sec61 translocon, polyubiquitylated, and then degraded by proteasomes (*40*). Most proteins that reach the ER probably follow this ERAD pathway, with only a small fraction passing scrutiny. This provides a "fail-safe" mechanism for ensuring that only functional and assembled proteins remain within the ER or pass onward. However, for some proteins, the overzealous ERAD pathway underlies a devastating pathology. In the cystic fibrosis transmembrane regulator (CFTR), a Cl^- channel, the point mutation most commonly associated with cystic fibrosis ($\Delta F508$) does not prevent the channel from functioning but does trigger ERAD-mediated degradation (*41*). The functional protein is thereby prevented from reaching the PM, where it is required to mediate Cl^- transport. The key point for this discussion is that most channels, which are invariably multimeric or pseudomultimeric (*8*), assemble into their functional complexes within the ER membrane.

All membrane proteins, with the exception of those in mitochondria and chloroplasts and most within peroxisomes, reach their final destination via the ER. The major routes are shown in Figure 1. Proteins destined for the inner nuclear membrane are probably recognized as they are synthesized in the ER, bind importin-α-16, and then pass directly from the ER to the outer nuclear membrane, which is continuous with the ER, and from there to the inner nuclear membrane via the nuclear pore complex (*42*). Some membrane proteins may be trafficked directly from the ER to peroxisomes in preperoxisome vesicles (*43*) and perhaps also to phagosomes (*44*), but all other membrane proteins pass through the Golgi en route to their destination. Here too, the ER exerts stringent quality control, retaining protein subunits until they assemble appropriately with partners. This is often achieved by having ER retention signals concealed only when the multimeric protein has properly assembled (*45–47*).

N-linked glycosylation, a feature of many membrane proteins, begins in the ER, and the modifications continue throughout the Golgi apparatus, assuming their final form before leaving the trans-Golgi network (TGN). From here, proteins are sorted into secretory vesicles or endosomes (*48*), and perhaps directly into secretory lysosomes (*49*). Secretory vesicles and early endosomes provide routes to the PM, where membrane fusion inserts proteins into the PM (*50*). Endocytosis, and in some cells phagocytosis, brings membrane proteins back from the PM into intracellular organelles, early endosomes and phagosomes, respectively. The latter are destined for lysosomes, where most proteins are degraded, but some proteins, like P2X4 receptors, protected by extensive glycosylation (*51, 52*), survive the hostile lysosomal environment and may later return to the PM by exocytosis from lysosomes (*51*). Early endosomes can deliver proteins back to the PM or, via late endosomes, to the Golgi or lysosomes (Figure 1).

Conserved motifs within membrane proteins play a large part in sorting, but additional mechanisms, like their membrane-spanning α-helices and association with other retained proteins, also contribute (*20, 21*). The sorting motifs are recognized by cytoplasmic coat proteins, which concentrate them into defined membrane regions and tag the region for onward

Table 1: Some Key Properties of Cell Membranes

	membrane thickness (nm)	$E_m{}^a$ (mV)	pH^b	redox potential^d	cholesterol:phospholipid ratio
mitochondria outer	2.13 (*253*)^e	0	7.2		0.06 (*254*)
mitochondria inner		140	8.5	− (*255*)	0.12 (*254*)
ER	3.75 (*256*)	∼0 (*88−90*)	7.2 (*257*)	++ (*258*)	0.065−0.237 (*254*)
Golgi	3.95 (*256*)	∼0 (*91*)	6.0−6.7 (*257*)		0.25 (*259*)
secretory vesicles	3.83 (*253*)^e	−76 (*92*)	5.2 (*257*)		
PM	3.56 (*256*) (basolateral), 4.25 (*256*) (apical)	−60	7.4^c	++ (*260*)	0.42 (*259*), 0.46−0.76 (*254*)
early endosomes			6.3−6.5 (*257, 261*)	+ (*262*)	0.80 (*259*)
late endosomes			5−6 (*257, 261*)	+ (*262*)	0.69 (*259*)
lysosomes	3.83 (*253*)^e	72 (*263*)	4.5−5.5 (*257, 261*)	+ (*262*)	0.38 (*264*)
phagosome	3.83 (*253*)^e	−27 (*265*)	8		
cytosol	−	−	7.2	− (*266*)	−

^{a}Typical membrane potential (cytosol relative to lumen or extracellular). ^{b}Typical luminal pH (*261*). ^{c}Typical plasma pH (*261*). ^{d}Redox state is shown with oxidizing (+) and reducing (−). ^{e}Estimated from ratios of membrane thickness relative to apical PM (*253*).

movement to a specific membrane. A cytosolic K(X)KXX sequence, for example, serves both to retain proteins within the ER and, after binding COPI coat protein, to return escapees to the ER from the cis-Golgi. Budding of the membrane from the donor organelle, transport of the resulting vesicle along microtubules or the actin cytoskeleton (*50, 53*), and fusion with the target organelle deliver the membrane protein to its next destination. Details of these processes, the essential role of small GTPases (*54*), the identities of the many sorting sequences (*32*), the role of lipids in these sorting events (*55*), and the behavior of the SNAREs that mediate vesicle fusion (*56*) are described elsewhere (*20, 21, 32*).

Even proteins destined to function in a specific membrane are dynamically shuffled between compartments: escaped ER proteins are trafficked back from the Golgi, a PM protein may be endocytosed and then reappear at the PM, and some proteins take very circuitous routes to their final destination. In polarized epithelia, for example, many proteins are directly dispatched to the apical or basolateral PM after being sorted at the TGN (*53*), but in hepatocytes, all PM proteins are first sent to the basolateral PM, from where apical proteins are selectively endocytosed and sent to the apical PM (*57*). These observations highlight the fact that most ion channels must pass through several different membranes to reach their destination and even then are likely to make periodic excursions into other membranes. How does the cell ensure effective control of Ca^{2+} channels as they pass through these different cellular compartments?

GETTING CA^{2+} CHANNELS HARMLESSLY TO THEIR DESTINATIONS

As proteins progress through membrane compartments, they are exposed to very different membrane and luminal environments. The lipid composition of the asymmetric bilayer differs in the different membranes (*58*), and this causes radical changes in both its physical (thickness, fluidity, curvature, etc.) and chemical properties (presence of polyphosphoinositides, etc.) (*59*) (Table 1). The membrane becomes thicker and more rigid between the ER, TGN, and PM as it comes to include more cholesterol and sphingolipids; phosphoinositides in the cytosolic leaflet also become more phosphorylated (*60*). Even within continuous membranes, subdomains (rafts, caveolae, etc.) that differ in their lipid and protein composition form (*58, 61*). As different proteins become segregated into the lumen and membranes, different organelles come to provide different luminal environments (proteins, pH, redox state, etc.), and they generate

different electrical potentials across the membrane (Table 1). All of these factors can affect the behavior of resident ion channels.

As the hydrophobic regions of membrane-spanning proteins snuggle into the hydrophobic bilayer, both adjust their structures: the bilayer locally adjusts its thickness, and the protein may modestly rearrange its structure (*59*). This "hydrophobic matching" is one of the mechanisms proposed to allow sorting of proteins into different membranes (*62*), but it also has functional consequences for ion channel activity: the bilayer is an "allosteric regulator of membrane function" (*59*). Nicotinic acetylcholine receptors, for example, come closest to mimicking their native behavior when reconstituted into vesicles in which the lipid composition resembles that of the PM (*63*). The single-channel conductance (γ) of Ca^{2+}-activated K^+ channels (BK channels) varies with membrane thickness (*64*), and IP$_3$R in the nuclear envelope and PM differ in their γ (*65, 66*). Many ion channels, including some Ca$_v$ channels, several P2X receptors, and many TRP proteins (*67*) require phosphatidylinositol 4,5-bisphosphate (PIP$_2$) for activity. These channels may be silenced by a lack of PIP$_2$ until they reach the PM. This brings us to a key question, and the focus of the next section of this review: how, as proteins pass through membrane compartments, does the cell cope with functional Ca^{2+} channels passing through many organelles before reaching their final destination? How, for example, do the ER, Golgi, and secretory vesicles cope with a fully assembled Ca$_v$ channel as it makes its way to its final destination in the PM?

The scale of the problem is evident from Table 2, which provides selected examples of Ca^{2+} channels and their usual distributions. The PM includes the greatest diversity of Ca^{2+} channels, and many appear to function exclusively within the PM. However, some channels function in more than one membrane: IP$_3$R, RyR, and TRPP2 (*47*) in the ER and PM (see later), or P2X4 receptors in the PM and lysosomes (*51*), for example. It is clear that for at least some of these channels, the same gene encodes the channel in each membrane compartment, but the extent to which post-transcriptional processing contributes to expression in different membranes is largely unresolved. In subsequent sections, we consider two issues: (1) the means whereby so many Ca^{2+} channels reach post-ER membranes, most commonly the PM, without perturbing the behavior of intervening organelles and (2) evidence that IP$_3$R and RyR, which are almost entirely expressed in the ER or sarcoplasmic reticulum (SR), are expressed in the PM of some cells.

Table 2: Distributions of a Selection of Ca^{2+}-Conducting Channels

channel	major location	additional locations	activating (inhibitory) regulation
IP$_3$R	ER	PM (65), Golgi (113), nucleus (115), secretory vesicles (118, 119)	IP$_3$, Ca^{2+}, ATP, cAMP, PKA, PKC, luminal chromogranin, Bcl-2, Bcl-X$_L$, RACK1, G$\beta\gamma$, NCS-1 (Mg^{2+}, Ca^{2+}, PIP$_2$, IRBIT, calmodulin, ankyrin, ERp44) (111, 198)
RyR	ER	PM (185), secretory vesicles (118, 169), endosomes (170)	Ca^{2+}, cyclic ADP ribose, ATP, Ca^{2+}-calmodulin-dependent protein kinase, PKA, FKBP12 (Mg^{2+}, Ca^{2+}, calmodulin) (199)
TPC1	lysosomes (233)	PM (267)	NAADP (233)
TRPP2	ER (47)	PM (47)	Ca^{2+}, casein kinase 2, α-actinin, ATP (cytosolic H$^+$) (268)
TRPM8	PM (269, 270)	ER (271)	cold, depolarization, PIP$_2$ (272) (spermine) (273)
TRPC7	PM (274)	TGN and Golgi (275)	G-protein-coupled receptors, diacyglycerol (276) (calmodulin, cytosolic Ca^{2+}) (277)
TRPV1	PM (278)	ER (78)	capsaicin, luminal H$^+$, heat, ATP, PKA, protein kinase C (75, 279) (PIP$_2$) (279)
TRPA1	PM (280)		cold, PIP$_2$ (281)
TRPML1	lysosomes, endosomes (282)	PM (283)	(Luminal H$^+$) (282)
Ca$_v$	PM	secretory vesicles (93)	depolarization, PIP$_2$, PKA, spermine (depolarization, arachidonic acid) (284, 285)
NMDA receptor	PM		glutamate, glycine, PIP$_2$ (extracellular: oxidation, H$^+$, Mg^{2+}, Zn^{2+}) (286, 287)
CNG channel	PM		cAMP, cGMP
P$_2$X4 receptor	PM	lysosomes (51)	ATP (extracellular H$^+$) (288)
pannexin-1	PM	ER (289)	depolarization, mechanical stress, Ca^{2+} (ATP, cytosolic H$^+$) (290)
connexins	PM		cytosolic Ca^{2+}, mechanical stress, depolarization (extracellular: Ca^{2+}, Mg^{2+}; cytosolic H$^+$) (290)
Orai1−3	PM		STIM1, extracellular Ca^{2+} (cytosolic Ca^{2+}) (147)
nicotinic receptor	PM		acetylcholine

GETTING CA^{2+} CHANNELS TO THE PLASMA MEMBRANE WITHOUT WAYWARD ACTIVITY

For many Ca^{2+}-permeable channels, avoiding wayward activity as they pass through different membranes is probably straightforward. Most ligand-gated ion channels, like nicotinic acetylcholine, ionotropic glutamate, and 5HT$_3$ receptors, open very infrequently in the absence of their agonist (68). They are presumably protected from activation in inappropriate organelles because the closed state is very stable and their ligand-binding sites are concealed within the lumen of the intermediate organelles. The Orai proteins that mediate SOCE are activated by interaction with stromal interaction proteins (STIMs), and this probably requires that the two proteins be in opposing membranes, the PM and ER, respectively (69). STIM1 is also expressed in the PM (70−72), from where it could conceivably activate Orai in transit through intracellular organelles, but STIM1 activates Orai only when it sheds Ca^{2+} from its luminal EF-hand, which would never occur for the extracellular EF-hand of STIM1 in the PM. Inappropriate activation of intracellular Orai is, therefore, probably prevented because its activation requires that STIM1 in the ER be presented to Orai in the PM at places where the two membranes are closely apposed.

However, for other Ca^{2+}-permeable channels, binding of a cytosolic ligand is sufficient to stimulate opening: cGMP or cAMP binding to cyclic nucleotide-gated (CNG) channels (73), or a decrease in the cytosolic Mg^{2+} concentration for

TRPM6 (74), for example. Several Ca^{2+}-permeable channels are activated by stimuli that freely cross membranes: capsaicin and related endogenous compounds are lipophilic and activate TRPV1 (75), and several TRP proteins (TRPV1−4, TRPM2, TRPM8, and TRPA) are activated by changes in temperature (76). It is hard to envisage how these channels can entirely avoid activation en route to the PM, although polymodal regulation of many channels, notably TRP channels (76), may provide some defense. It is perhaps not surprising, therefore, that some of these channels destined for the PM, including TRPM8 (77), TRPV1 (78), and TRPP2 (47), do mediate release of Ca^{2+} from intracellular stores. The physiological significance of the activities of these channels "in transit" is generally unresolved, but the differential distribution of TRPM8 between the PM and ER has been implicated in the progression of prostate cancer (77).

For the remainder of this section, we have chosen to examine a specific example, Ca$_v$ channels, and consider their likely behavior as they pass from the ER through the Golgi and secretory vesicles to the PM, where most are located and where their opening is regulated by membrane potential and numerous modulatory signals (79). These important channels are almost entirely responsible for transducing changes in membrane potential into signals (increases in cytosolic Ca^{2+} concentration) that can regulate intracellular activities. Trafficking of some Ca$_v$ channels (Ca$_v$1 and Ca$_v$2) to the PM is facilitated by association of the pore-forming α_1 subunits with auxiliary Ca$_v\beta$ subunits, which

may mask an ER retention signal within the α_1 subunits (*80*). Mutations within the α_{1A} subunit of the $Ca_v2.1$ channel trap associated subunits within the ER and gives rise to episodic ataxia type 2 (*81*). Whether the pathology reflects aberrant activity of $Ca_v2.1$ within the ER or loss of activity at the PM is unresolved, but in either case, the importance of effective trafficking to the PM is clear.

Members of each of the three major families of Ca_v channels [Ca_v1 (L-type), Ca_v2 (N-, P/Q-, and R-type), and Ca_v3 (T-type)] are inactivated (at very different rates) by sustained depolarization to potentials of approximately -60 to -10 mV (Ca_v1), -120 to -20 mV (Ca_v2), and -100 to -60 mV (Ca_v3) (*8*, *82*). At the resting membrane potential of the PM (typically approximately -60 mV), most of these channels will not be inactivated and can open during transient depolarizations. Many ion channels, including Ca_v channels (*83*), CNG channels (*84*), and glutamate receptors (*85*, *86*), are blocked by polyamines, like spermine, and can mediate ion fluxes only when changes in membrane potential dislodge the polyamine. For Ca^{2+}-permeable AMPA receptors, an accessory protein, stargazin, both guides the receptors to the PM and relieves the block by polyamines (*87*), perhaps thereby ensuring effective block of the channels until they leave the ER. The important point is that activation of Ca_v channels requires transient depolarization from a hyperpolarized membrane potential for activation. However, the potential across the membranes of the ER (*88–90*) and Golgi (*91*) is probably close to 0 mV, sufficient to inactivate most Ca_v channels. However, secretory vesicles appear to maintain a membrane potential more like that of the PM (-76 mV) (*92*), and with individual vesicles showing considerable variability in membrane potential (-11 to -160 mV), it seems likely that Ca_v channels within secretory vesicles may be exposed to potential changes that would allow their activation. Because secretory vesicles, in common with every other intracellular Ca^{2+} store, contain only a limited pool of Ca^{2+}, Ca_v within them could contribute to Ca^{2+} signaling only if bouts of activation were interspersed with periods of closure to allow the vesicles to refill with Ca^{2+}. The possibility that Ca_v channels might be activated within secretory vesicles assumes further significance in light of evidence that a large reservoir of functional Ca_v channels may be retained within the secretory vesicles of neuroblastoma cells, from where trafficking to the PM is dynamically regulated (*93*). Whether other factors, like low pH or the absence of PIP_2 (see below), silence Ca_v channels in secretory vesicles or whether they are active and responsive to changes in vesicle membrane potential deserves further study.

The pH to which the luminal/extracellular surface of membrane proteins is exposed also changes dramatically as they are transported from the ER to the PM (Table 1). Many Ca^{2+} channels, including Ca_v1 (*94*), CNG channels (*95*), TRPV5, and TRPV6 (*96*), are inhibited by the low pH encountered within the secretory pathway. Other Ca^{2+} channels, such as TRPV1 (*97*), TRPM6 (*98*), and TRPM7 (*99*), are stimulated by this low pH, although this may be accompanied by a reduced Ca^{2+} flux as H^+ within the permeation pathway attenuates Ca^{2+} binding and increases the relative permeability to monovalent cations (*99*).

The different lipid compositions of the PM and earlier membranes (Table 1) may also restrain the activity of Ca^{2+} channels in transit. PIP_2 is enriched in the inner leaflet of the PM and is essential for the activity of Ca_v2 channels (*100*). The activity of Ca_v1 channels is enhanced by cholesterol (*101*), the concentration of which increases as membranes progress toward the PM (Table 1). Similar interactions may inhibit the activities of

other Ca^{2+} channels until they reach the PM: many TRP channels are stimulated by PIP_2 (*102*) or the diacylglycerol produced by its hydrolysis (*103*), and the activities of CNG channels (*104*) and TRPC3 (*105*) are enhanced by cholesterol.

Finally, Ca_v and many other Ca^{2+} channels have their responses tuned by association with additional proteins, such that successful targeting of these proteins to the PM can unmask latent channel activity. We have already discussed the role of $Ca_v\beta$ subunits in coordinating PM targeting and the activity of Ca_v channels (*80*). Palmitoylation of Ca_v channels likewise both regulates their trafficking to the PM and enhances their activity (*106–108*). Anchoring of cyclic AMP-dependent protein kinase (PKA) at the PM by A-kinase-anchoring protein (AKAP15) positions PKA close to Ca_v1 channels, allowing phosphorylation to increase their activity (*109*).

Clearly, Ca_v channels can reach the PM without overly perturbing cytosolic or luminal Ca^{2+} regulation as they pass through intervening organelles. A few general themes emerge. Ca_v (and other) channels in transit may be inactive because the intervening environment is hostile (e.g., inactivation by sustained depolarization, lipids, pH, etc.). A single modification (e.g., association with a β-subunit or palmitoylation) may both enhance activity and simultaneously provide an express ticket to the PM, thereby ensuring that the most active channels reach the PM without lingering in the ER. Additional PM proteins (e.g., AKAP-anchored PKA) may enhance the activity of juxtaposed channels. Whether these, or additional mechanisms, are wholly effective in silencing Ca_v channels in transit seems to be unresolved. Do Ca_v channels, for example, mediate voltage-gated Ca^{2+} fluxes in secretory vesicles? Do they, or other Ca^{2+} channels in transit, contribute to the unresolved leak of basal Ca^{2+} from the ER? What are the physiological roles of those Ca^{2+} channels in transit that are not completely silenced as they make their way to the PM?

COUNTING IP₃ RECEPTORS INTO THE PLASMA MEMBRANE

Most IP_3R in most cells are expressed within ER membranes, or the nuclear envelope, which is continuous with them (*110*, *111*). This pattern of expression is maintained by several ER retention signals within the six C-terminal transmembrane domains of IP_3R (*112*). Nevertheless, there is persuasive evidence that functional IP_3R can also be expressed within the Golgi apparatus (*113*, *114*), the nucleoplasmic reticulum (*115*), and, more contentiously, secretory vesicles (*116–119*). In this section, we consider evidence that IP_3R, the archetypal intracellular Ca^{2+} channels, are also functionally expressed in the PM of some cells, notably DT40 cells, a prelymphocyte cell line (*120*), and mammalian B-lymphocytes (*65*).

Several studies had suggested that IP_3R might be expressed within the PM (reviewed in refs *65* and *121*), but the results were inconclusive and in many cases likely to reflect the activities of intracellular IP_3R. Subcellular fractionation showed IP_3R to copurify with PM markers (*122–124*), probably because IP_3R within the ER are closely associated with the PM (*123*, *125*). The same explanation might account for the presence of IP_3R immunostaining close to the PM of oviductal ciliated cells (*126*). Cell surface labeling suggested the presence of IP_3R within the PM (*127–130*). Patch-clamp recording identified IP_3-activated, Ca^{2+}-permeable channels in the PM of some cells (*131–136*), and a protein purified from a liver PM fraction behaved as an

IP$_3$-gated channel when reconstituted into lipid bilayers (*137*). However, the electrophysiological properties of these channels were very different from those of IP$_3$R either in nuclear membranes (*66, 110, 111*) or after reconstitution of intracellular IP$_3$R into bilayers (*138, 139*). With the exception of IP$_3$R in olfactory cilia (*131*), which differ from intracellular IP$_3$R in their sensitivity to ruthenium red, insensitivity to ATP, and lower conductance, the other reports of IP$_3$-activated PM channels failed to establish whether the channels were actually IP$_3$R within the PM or more likely other channels indirectly activated by stimulation of IP$_3$R within the ER. Our work with DT40 cells (*65, 140*) and subsequent work by others (*141*) have provided conclusive evidence that functional IP$_3$R can be expressed in the PM of at least some cells.

Stimulation of DT40 cells with either anti-IgM to activate the B-cell receptor (BCR) or trypsin to activate the protease-activated receptor (PAR) leads to activation of phospholipase C, formation of IP$_3$, and thereby release of Ca^{2+} from intracellular stores mediated by IP$_3$R (*120, 142*). DT40 cells are uniquely suited to analyses of these signaling pathways because Kurosaki and his colleagues generated a DT40 cell line (DT40-KO cells) in which the genes for all three IP$_3$R subtypes are disrupted (*142*). DT40-KO cells are the only vertebrate cells unequivocally devoid of functional IP$_3$R and therefore the only null background in which recombinant IP$_3$R can be functionally expressed free of the complexity arising from association with residual native IP$_3$R (*120, 143*). The inability of either anti-IgM or trypsin to evoke Ca^{2+} release in DT40-KO cells and restoration of that response by expression of recombinant IP$_3$R firmly establishes the role of the IP$_3$R in mediating Ca^{2+} release (*65*).

The same stimuli that evoke release of Ca^{2+} also evoke entry of Ca^{2+} across the PM of DT40 cells. In most cells, such Ca^{2+} entry is commonly mediated by SOCE (*144, 145*). The defining feature of SOCE is that depletion of intracellular Ca^{2+} stores provides a sufficient stimulus for its activation (*144*). Thapsigargin, which inhibits SERCA and thereby causes loss of Ca^{2+} from the ER without engaging the signaling pathways used by receptors, is often used to activate SOCE (Figure 2A). In many cells, the electrophysiological manifestation of SOCE is the Ca^{2+} release-activated current (I_{CRAC}), which is characterized by its inward rectification, Ca^{2+} selectivity, and low unitary conductance, block by low concentrations of Gd^{3+}, and activation by depleted Ca^{2+} stores (*146, 147*). The molecular architecture of the SOCE pathway has recently been established (*13*), with STIM1 emerging as the luminal Ca^{2+} sensor in the ER membrane (*148, 149*), and Orai proteins as the pore-forming subunits of the channel in the PM (*150*). After Ca^{2+} is lost from the ER, the luminal EF-hand of STIM1 loses Ca^{2+}, causing it to cluster. The clustered polybasic C-termini of STIM1 can then interact with PIP$_2$ in the PM, allowing a stretch of conserved residues within the cytosolic tail of STIM1, the CRAC activation domain (CAD), to stimulate opening of Orai (*69, 151*). Further details of the mechanisms of activation of SOCE (*13*), including the possible involvement of TRP proteins (*152*), the possibility that biochemical signals might link STIM1 to activation of Orai (*153*), the relative roles of STIM1 and STIM2 (*154, 155*), and the possibility that STIM might activate Orai by causing its assembly into a tetrameric channel (*156*), are discussed elsewhere.

SOCE, with the properties of I_{CRAC} (*157, 158*) and mediated by STIM1 (*158*) and Orai1/2 (*159*), is expressed in DT40 cells, and it can be activated by thapsigargin (*65, 120, 159*) (Figure 2A). However, whereas this thapsigargin-activated SOCE is entirely blocked by low concentrations of Gd^{3+} (*65*) or La^{3+} (*159*), the Ca^{2+} entry evoked by activation of the BCR is only partially blocked: typically, ~50% of the Ca^{2+} entry evoked by the BCR appears not to be mediated by SOCE (Figure 2A) (*65, 159*). Both components of Ca^{2+} entry require functional IP$_3$R: activation of the BCR evokes neither Ca^{2+} release nor Ca^{2+} entry in DT40-KO cells (*65, 121, 142, 159*). Others have recently suggested that a non-SOCE pathway in DT40 cells is evoked only by activation of the BCR, that the need for IP$_3$R is limited to its ability to empty intracellular Ca^{2+} stores, and that activation of the pathway requires a BCR-evoked signal and STIM1, but not Orai1 (*159*). These observations together with earlier work suggesting an essential role for IP$_3$R in Ca^{2+} entry that was independent of its ability to conduct Ca^{2+} (*160*) and evidence implicating TRPC7 in the non-SOCE pathway (*161*) have added complexity and considerable confusion to our understanding of Ca^{2+} entry in DT40 cells (*120*). Here we focus on evidence that DT40 cells express functional IP$_3$R in the PM and suggest that these may mediate Ca^{2+} entry via the non-SOCE pathway.

In whole-cell patch-clamp recording from DT40 cells, IP$_3$ stimulates opening of very small numbers of large-conductance cation channels (1.9 ± 0.2 channels/cell). These channels are absent from DT40-KO cells. They are inactive in cells stimulated with IP$_3$ in the presence of its competitive antagonist, heparin. They are activated by adenophostin A, another agonist of IP$_3$R. Like intracellular IP$_3$R, they are also regulated by ATP and Ca^{2+} (*65*) (Figure 2B). Furthermore, in common with the non-SOCE Ca^{2+} entry pathway, the IP$_3$-activated PM channels are insensitive to concentrations of Gd^{3+} that block SOCE. Expression in DT40-KO cells of either IP$_3$R1 or IP$_3$R3 restores both the non-SOCE Ca^{2+} entry pathway and the IP$_3$-activated PM channels. However, expression of IP$_3$R1 with a point mutation in the putative pore (D2550A) that prevents IP$_3$-evoked Ca^{2+} release fails also to restore either Ca^{2+} entry mediated by the non-SOCE pathway (but see ref *160*) or IP$_3$-activated PM cation channels (*65*). Similar channels are detected in perforated-patch recordings from DT40 cells after stimulation of the BCR, and these channels are activated with a time course similar to that of the Gd^{3+}-insensitive Ca^{2+} entry evoked by the BCR in intact cells. Collectively, these results demonstrate that the PM channels activated by IP$_3$ do not reflect further activation of SOCE, which might have resulted from more complete emptying of intracellular Ca^{2+} stores by IP$_3$: the channels are Gd^{3+}-insensitive, their electrophysiological properties are entirely different from I_{CRAC}, and IP$_3$ activates the channels similarly before or after store depletion (*65*). The coincident behaviors of the IP$_3$-activated PM channels and the Ca^{2+} signals mediated by the non-SOCE pathway further suggest that the latter might be mediated by the PM channels. A key question, however, is the identity of the PM channels: are they IP$_3$R working within the PM, or are they other channels activated via IP$_3$ binding to IP$_3$R in their conventional location, the ER?

Several lines of evidence provide conclusive evidence that IP$_3$R are themselves functional within the PM. Mutation of residues within the putative pore of IP$_3$R1 causes changes in the unitary conductance (γ) that can be easily resolved by patch-clamp recording from intracellular IP$_3$R within the outer nuclear envelope of DT40 cells (*66, 110*). The latter is continuous with the ER and allows single-channel recording from intracellular channels that would otherwise be inaccessible. The important point is that the same mutations cause similar changes in γ for intracellular IP$_3$R and for IP$_3$-activated cation channels in the
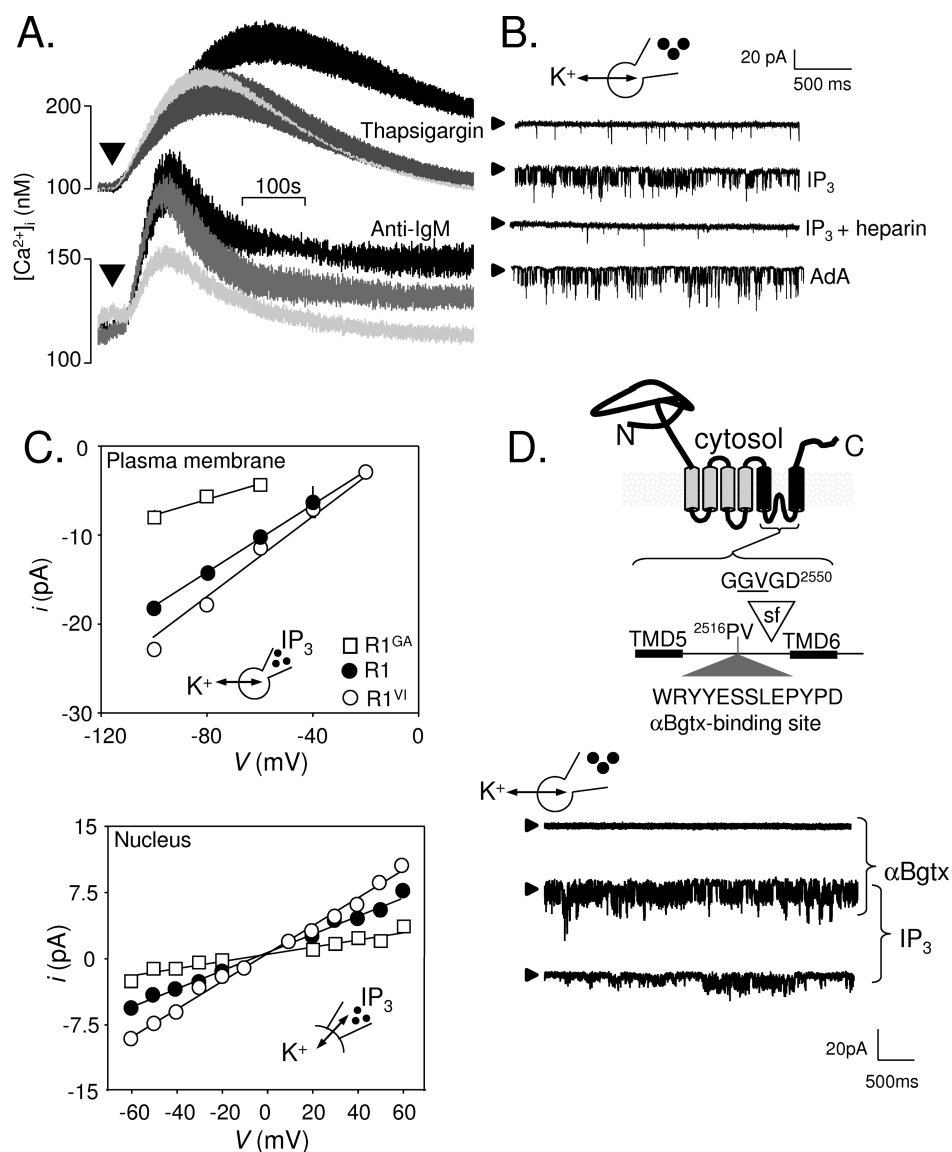
FIGURE 2: Expression of IP$_3$ receptors in the plasma membrane of DT40 cells. (A) Thapsigargin (top) stimulates Ca^{2+} release (palest line, in Ca^{2+}-free medium) and Ca^{2+} entry (black line, in Ca^{2+}-containing medium). The latter, SOCE, is entirely blocked by 300 nM GdCl$_3$ (gray line). The bottom panel shows that activation of the BCR with anti-IgM stimulates both Ca^{2+} release and Ca^{2+} entry, but the latter is only partially inhibited by GdCl$_3$. (B) Whole-cell patch-clamp recoding from DT40 cells with IP$_3$, IP$_3$ with heparin, or adenophostin A in the patch pipette. The holding potential was -100 mV; arrowheads denote the closed state. (C) Current$-$voltage ($i-V$) relationships for the IP$_3$-stimulated currents recorded from the PM or nuclear envelope of DT40-KO cells stably transfected with wild-type IP$_3$R1 (R1) or IP$_3$R1 with mutants in the putative pore (G2547A, R1^{GA}; V2548I, R1^{VI}). The point mutations similarly affected γ_K of the IP$_3$-activated currents in both settings. (D) The six TMDs of a single IP$_3$R subunit are shown to highlight the putative selectivity filter (sf) and the engineered αBgtx-binding site. In whole-cell patch-clamp recordings from DT40 cells expressing IP$_3$R1 with this αBgtx-binding site, intracellular IP$_3$ stimulated channel openings, and both P_o and γ_K were increased by extracellular αBgtx. Reproduced from ref *65* with permission. Copyright 2006. American Academy for the Advancement of Science.

PM (*65, 141*) (Figure 2C). Clearly, if the effects of IP$_3$ on the PM were mediated solely by its interaction with intracellular IP$_3$R, pore mutations within the IP$_3$R would not be expected to affect the γ of the PM channels. We also introduced a binding site for α-bungarotoxin (αBgtx) into the luminal loop linking the last pair of transmembrane domains (TMD5$-$6) of IP$_3$R1 and expressed it in DT40-KO cells (Figure 2D). From the known topology of the IP$_3$R, we expected the αBgtx-binding site to be luminal for IP$_3$R within the ER, but extracellular for IP$_3$R expressed in the PM. In whole-cell patch-clamp recordings from DT40 cells expressing these IP$_3$R, intracellular IP$_3$ again activated cation channels in the PM, but their γ and open probability (P_o) were both increased by addition of extracellular, but not intracellular, αBgtx (*65*). These results establish that the recombinant IP$_3$R must straddle the PM. Finally, others have expressed IP$_3$R with

mutations in sites that are phosphorylated by PKA and shown that the P_o of the PM cation channels activated by adenophostin A is increased by forskolin (to activate PKA) for wild-type IP$_3$R; IP$_3$R with phospho-mimetic mutations are hyperactive, and those with mutations that prevent phosphorylation have much reduced activity (*141*). Collectively, these results demonstrate unequivocally that functional IP$_3$R are expressed in the PM of DT40 cells. We stress, however, that IP$_3$R are not expressed in the PM of all cells; we have not, for example, detected IP$_3$R in the PM of either Sf9 or human embryonic kidney (HEK) cells.

Functional IP$_3$R in the PM of DT40 cells are expressed at an exceptionally low density, just 1.9 ± 0.2 channels/cell (*65*), and we have never, even in cells massively overexpressing intracellular IP$_3$R, detected more than five functional IP$_3$R in the PM. There are some additional examples of cells expressing such tiny
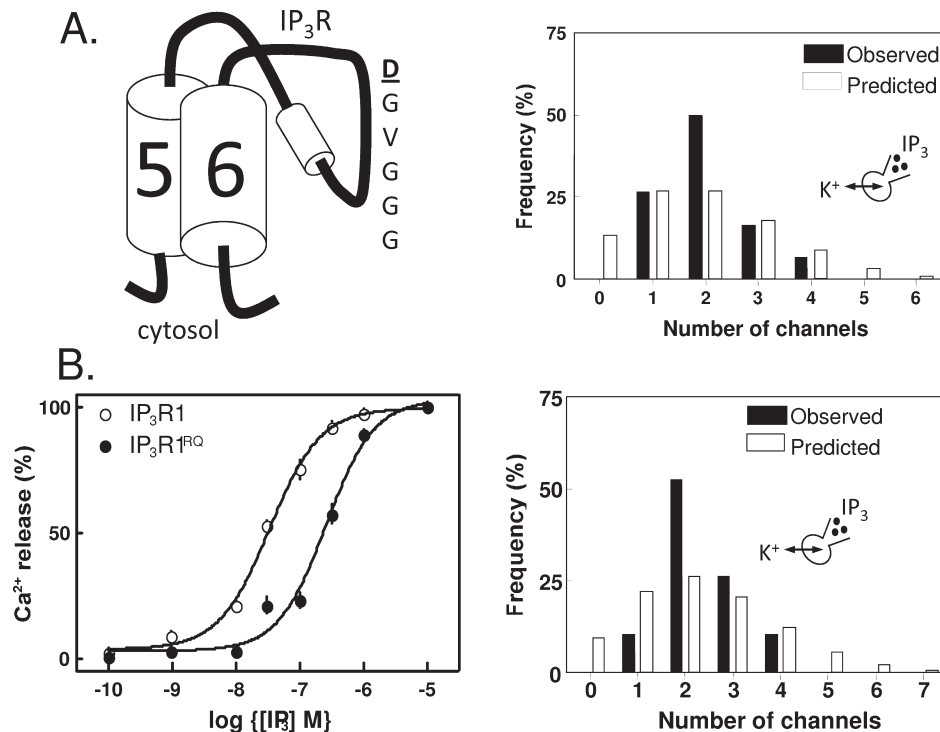
FIGURE 3: Counting IP_3 receptors into the plasma membrane. (A) A point mutation within the putative pore region of IP_3R1 (D2550A, highlighted) causes luminal/extracellular Ca^{2+} to block the channel, but it does not prevent cells from reliably counting IP_3R into the PM. The histogram shows the observed and predicted (from the Poisson distribution) numbers of functional IP_3R detected in each cell and establishes that IP_3R are not randomly inserted into the PM. (B) A point mutation within the IP_3-binding core (R568Q, IP_3R1^{RQ}) reduces the binding affinity of the IP_3R for IP_3 by 10-fold, evidenced by radioligand binding analyses (not shown) and the 10-fold decrease in the sensitivity of Ca^{2+} release to IP_3 (left). The reduced sensitivity to IP_3 does not impair the reliability with which IP_3R are functionally expressed in the PM (right). Reproduced with permission from ref *140*. Copyright 2008. American Society for Biochemistry and Molecular Biology.

numbers of channels in the PM, ~10 intermediate conductance Ca^{2+}-activated K^+ channels ($IK_{Ca}1$) in a resting T-cell (*162*), for example, or two or three RyR-like channels in rat portal vein myocytes (*163*) (see later), but most channels expressed in the PM occur at much higher densities (typically > 100 channels/μm^2 or several thousand channels per cell) (*8*). An intriguing feature of IP_3R expression in the PM is that in several hundred recordings, we never failed to detect at least one IP_3R in the PM (*65, 140*). If these IP_3R had been randomly inserted into the PM with very low probability, perhaps reflecting "leakage" from the ER, for example, we would have expected (on the basis of the Poisson distribution) ~28% of cells to lack PM IP_3R. These results suggest that functional IP_3R are reliably counted into the PM. This may reflect either counting of proteins into the PM or a regulatory process that only ever allows very few of a larger number or resident channels to be active within the PM.

In seeking to address mechanisms that might allow such reliable counting of PM IP_3R, we considered that feedback regulation of IP_3R trafficking to the PM from active IP_3R within the PM was the most likely possibility. An IP_3R in which a single residue (D2550A) was mutated to create a pore that is blocked whenever its luminal surface is bathed in Ca^{2+} provides a channel that is effectively "pore-dead" throughout its normal life cycle as it passes from ER to PM, yet these channels were effectively counted in appropriate numbers (~2 IP_3R/cell) into the PM (Figure 3A). Another possibility was that a feedback signal arose from an earlier step in the activation of IP_3R by IP_3, a conformational change in the IP_3-binding site, for example. We therefore expressed a mutant IP_3R (R568Q) in which the affinity for IP_3 was reduced by ~10-fold, arguing that at resting levels of IP_3 such IP_3R would now be only 10% as likely as normal IP_3R

to bind IP_3, but these IP_3R, like those that were pore-dead, were also reliably counted into the PM with ~2 IP_3R/cell (Figure 3B) (*140*). These rather puzzling results suggest that very small numbers of functional IP_3R are reliably counted into the PM in the apparent absence of any obvious feedback regulatory mechanism.

FUNCTIONAL RYANODINE RECEPTORS IN THE PLASMA MEMBRANE

RyR are the closest relatives of IP_3R, with which they share both structural (*164, 165*) and functional properties (*166*), and like IP_3R, they are expressed predominantly within the membranes of the ER (or SR in muscle), where they are retained by ER retention signals within the TMDs (*167*). However, as with IP_3R, evidence that RyR may also be expressed in post-ER membranes, and in the PM of some cells, is accumulating.

Within pancreatic β-cells, for example, or insulinoma cells derived from them, functional RyR appear to be expressed within the membranes of insulin-containing secretory vesicles (*168, 169*) and/or endosomes (*170*), whereas neither organelle expresses IP_3R (*171*). In chromaffin cells, both RyR and IP_3R appear to escape the ER and function as Ca^{2+} release channels within secretory vesicles (*118*). Perhaps more contentious is the possibility that RyR may be expressed in the PM (*172*). In this section, we briefly review the evidence that RyR are functionally expressed in the PM of some cells, and in the concluding section, we consider the possible physiological significance of RyR and IP_3R in the PM.

An early suggestion that RyR might be expressed in the PM came from studies of osteoclasts (*173*). Osteoclasts probably

express functional RyR. These may, as they do elsewhere, amplify, by Ca^{2+}-induced Ca^{2+} release, the Ca^{2+} signals generated by activation of other Ca^{2+} channels within intracellular stores or the PM (*174, 175*). However, the evidence that RyR are also expressed in the PM of osteoclasts deserves close examination because the conclusion is important. Extracellular ruthenium red, an antagonist of RyR, blocked Ni^{2+}-evoked Ca^{2+} signals (*173*). Ni^{2+} is known to activate the extracellular Ca^{2+}-sensing receptor (CaR), a G-protein-coupled receptor (*176*), although the role of CaR in osteoclasts has been contentious. The inhibition by ruthenium red of Ni^{2+}-evoked Ca^{2+} signals need not reflect an action at PM RyR, because ruthenium red is membrane-impermeant and its most substantial effects on RyR appear to be mediated by binding to its cytosolic surface (*177*). It is perhaps more likely that ruthenium red, a polycation, interacts with the Ca^{2+}-sensing receptor (CaR), which is known to be regulated by polyvalent metal ions and polyamines (*176*). This interpretation would also be consistent with evidence that ruthenium red itself evoked Ca^{2+} signals in osteoclasts (*178*). Ni^{2+} was suggested, although without quantitative analysis, to inhibit binding of [^{3}H]ryanodine to osteoblasts (*173*), but these binding assays do not distinguish intracellular RyR from those in the PM. Because ryanodine binding is use-dependent, any effect of agents that increase the cytosolic Ca^{2+} concentration on [^{3}H]ryanodine binding might simply reflect regulation of intracellular RyR by Ca^{2+}. Confocal microscopy identified peripheral immunostaining for RyR (*173*), but the resolution is insufficient to resolve whether RyR were in the PM or within ER lying close to it. An anti-peptide antiserum (Ab[129]) raised to 22 residues within a region toward the C-terminus of RyR2 immunostained intact osteoclasts (*173*). Because the N-terminus of the RyR is cytosolic (*179*), the limited information provided in the original publication (*173*), namely that the epitope lies between TMD6 and TMD7 of the originally proposed 10-TMD model of RyR (*180*), would place the epitope within the cytosol. Subsequent work revealed that Ab[129] was raised to residues 4676−4699 of rabbit RyR2 (*181*), and the revised six-TMD models of RyR (*167, 179*) would place the sequence between TMD2 and TMD3 (*181*), which again places the epitope within the cytosol. Immunostaining of intact cells with this antiserum is therefore unlikely to reflect its reaction with a PM RyR. Subsequent studies, using valinomycin to manipulate membrane potential, indicated that hyperpolarization increased the susceptibility to blockade by ryanodine of the Ca^{2+} release evoked by Ni^{2+} (*182*). This was interpreted as evidence that the RyR might sense membrane potential (*182*) either directly (because it was resident within the PM) (*175, 178*) or indirectly via conformational coupling to another PM voltage sensor. Evidence that other G-protein-coupled receptors are regulated by membrane potential (*183*) raises the possibility that the voltage sensor might even be the CaR itself. The significance of the latter findings (*182*), which provide no decisive evidence of a functional RyR in the PM, is diminished by the observation that in osteoclasts not treated with valinomycin, responses to Ni^{2+} were insensitive to ryanodine (*182*). Finally, patch-clamp recordings from excised patches of the PM of osteoclasts resolved small numbers of Ba^{2+}-permeable channels with unexpectedly high P_o values (0.5−0.95) in the absence of any known agonists of RyR, but that activity was massively attenuated by application of cytosolic ruthenium red or an antiserum to a cytosolic epitope of the RyR (*175*). Neither agent is likely to be specific, although the observation that both inhibit the active channels is suggestive of a RyR, but

the results are perplexing. The authors show that with symmetrical Ba^{2+}-containing solutions, the reversal potential is (as might be expected) 0 mV, but most traces, including those with massive channel activity, show excised patches at a holding potential of 0 mV using symmetrical Ba^{2+}-containing solutions. Under these conditions, there should be no currents. The inescapable conclusion is that the electrophysiological evidence in support of RyR in the PM of osteoclasts lacks credibility (*175*). We suggest that although Zaidi and his colleagues were among the first to suggest that RyR might be expressed in the PM (*173*), neither the original nor subsequent reports provide compelling evidence.

High-conductance PM cation channels resembling RyR have been detected in other cell types. In smooth muscle from portal vein, for example, caffeine, ryanodine, Ca^{2+}, or extracellular stimuli that evoke an increase in cytosolic Ca^{2+} concentration stimulated opening of high-conductance ($\gamma_K \sim 190$ pS) cation channels that were permeable to Ca^{2+} and Ba^{2+}, and only modestly selective between cations ($P_{Ca}/P_{Na} \sim 21$) [although more so than conventional RyR (*184*)] (*163*). It is also noteworthy that the γ of these PM channels is substantially lower than that of RyR reconstituted into lipid bilayers (*184*) but similar to that of RyR in the PM of insulinoma cells ($\gamma_K \sim 169$ pS) (*185*) (see later). In whole-cell recordings, no more than three simultaneous openings of these channels were detected, suggesting that very few were expressed in the PM. These authors may be correct in cautiously concluding that the PM channels are not RyR themselves, but other Ca^{2+}-sensitive channels activated in response to Ca^{2+} released from intracellular RyR (*163*), but it remains entirely plausible that these channels result from RyR within the PM that are regulated by physiological stimuli (norepinephrine or acetylcholine) (*163*). Other studies of gastric (*186*) or arterial (*187*) smooth muscle detected caffeine-stimulated nonselective cation channels in the PM, but their insensitivity to ryanodine (*186*), low conductance, or lack of permeability to bivalent cations (*187*) suggests that these are probably not RyR in the PM. In cardiac myocytes, too, caffeine or an increased cytosolic Ca^{2+} concentration stimulated opening of cation channels with high conductance ($\gamma_{Na} \sim 400$ pS) and permeability to Ba^{2+} (*188, 189*). The most persuasive evidence that these might be RyR in the PM is the ability of ryanodine, as it does for RyR in bilayers (*172, 177*), to lock the channels into a subconducting state (*189*). Collectively, these observations provide persuasive evidence that cardiac myocytes may express just one to four functional RyR within the PM (*189*). The possibility that ventricular myocytes express small numbers of RyR in the PM clearly deserves further study both to strengthen the conclusion and to address likely physiological roles.

Our analyses of PM RyR2 have concentrated on RINm5F insulinoma cells (*185*), where 4-chloro-*m*-cresol (4CmC), at concentrations likely to be selective for activation of RyR1 and RyR2, evokes both Ca^{2+} release and Ca^{2+} entry, the latter via a pathway that appears not to involve SOCE. Both responses are blocked by inactivating RyR by prior incubation with ryanodine (Figure 4A). From PCR analyses, IP$_3$R (almost entirely IP$_3$R3) are the major ($\sim$97%) intracellular Ca^{2+} channels in RINm5F cells, but they also express RyR (almost entirely RyR2). After subcellular fractionation, IP$_3$R3 is concentrated in the ER-enriched fractions, while $\sim$20% of RyR2 is concentrated in fractions that include the PMCA (i.e., they include PM) but exclude IP$_3$R3. In both cell-attached and excised patch-clamp recordings from RINm5F cells, 4CmC and caffeine activate
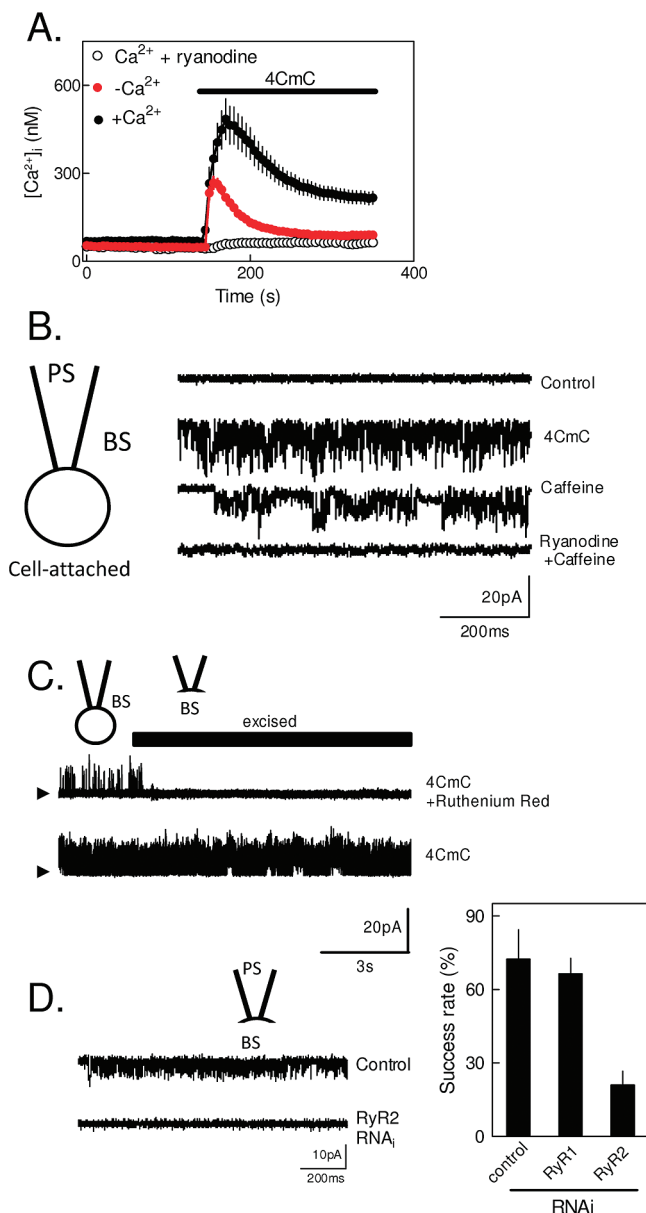
FIGURE 4: Functional ryanodine receptors in the plasma membrane of RINm5F insulinoma cells. (A) Ca^{2+} signals evoked in populations of cells by 4CmC (1 mM) with or without prior treatment with ryanodine (400 μM) and in either normal or Ca^{2+}-free medium. The results are consistent with activation of RyR causing both Ca^{2+} release and Ca^{2+} entry. (B) Cell-attached recordings from cells with cesium methanesulfonate in both bathing (BS) and pipette (PS) solutions at a holding potential of −100 mV. Caffeine (1 mM), 4CmC (1 mM), or ryanodine (400 μM) was included in BS as indicated. Arrowheads denote the closed state. (C) 4CmC activates channels in the cell-attached mode, which are then rapidly inhibited when the patch is excised into BS containing the membrane-impermeant inhibitor of RyR, ruthenium red (10 μM). (D) Selective inhibition of RyR2 expression using RNAi attenuates the electrical activity evoked by 4CmC. Typical records for control and RyR2-RNAi-treated cells are shown, and the success rate for detecting 4CmC-activated channels in the PM is shown for mock-transfected cells or cells transfected with RNAi for RyR1 or RyR2. Reproduced with permission from ref *185*. Copyright 2009. American Society for Biochemistry and Molecular Biology.

relatively nonselective, high-conductance (γ_K = 169 pS) cation channels that are inhibited by prior incubation with ryanodine (Figure 4B). The same channels are inhibited by the membrane-impermeant inhibitor of RyR, ruthenium red, when it is applied

to the cytosolic surface of the RyR, but not when applied to the extracellular surface (Figure 4C). There seem, although the estimate is only approximate, to be ~10 of these channels per cell. We (*185*) and others (*172*) have noted that neither γ of these channels nor the effects of high concentrations of ryanodine on γ faithfully replicate the exhaustively studied behavior of RyR in lipid bilayers. RyR in bilayers typically have γ values for K^+, Na^+, and Cs^+ that are 3−4-fold larger than those observed in our recordings from the PM (*185*) (practical difficulties have prevented us from measuring γ values for bivalent cations of the PM channels), and ryanodine usually locks RyR into a subconducting state (*190*). These disparities raise the possibility that the PM channels, while sharing key permeability properties and considerable pharmacology with RyR (activation by caffeine, 4CmC; inhibition by ryanodine and cytosolic ruthenium red; insensitivity to nifedipine and clotrimazole), may reflect the activity of an unrelated channel. Alternatively, it may be, as has been reported for IP_3R (*111*), that the behavior of RyR in a native membrane (the PM) is not faithfully replicated in a synthetic lipid bilayer. Our most direct evidence that the 4CmC-activated channels detected in the PM are RyR2 is provided by RNAi, which selectively attenuated expression of IP_3R3, RyR1, or RyR2 by ~60−70% (*185*). Loss of RyR2 alone attenuated the Ca^{2+} signals and Mn^{2+} entry evoked by 4CmC and substantially reduced (though it did not abolish) the detection of 4CmC-activated cation channels in the PM (Figure 4D) (*185*). Collectively, these results provide persuasive evidence that RyR2 is functionally expressed in the PM of RINm5F insulinoma cells. At present, we have only preliminary data to suggest that primary pancreatic β-cells might also express RyR in the PM (*185*).

Three issues arise from evidence that at least some cells might express functional RyR in the PM. First, how do RyR, with multiple determinants of ER retention (*167*), selectively escape the ER to reside, in small numbers, in the PM? Second, all electrophysiological analyses of RyR have hitherto used proteins reconstituted into lipid bilayers because the ER and SR where most native RyR reside are, for all practical purposes, inaccessible to patch-clamp recording. RyR in the PM provide opportunities to explore their behavior with patch-clamp techniques in a native membrane. Third, and the topic of the final section, what are the physiological roles of RyR and IP_3R in the PM?

PHYSIOLOGY OF "INTRACELLULAR" CA^{2+} CHANNELS IN THE PLASMA MEMBRANE

Within their usual setting, the membranes of the ER or SR, Ca^{2+} is probably the only permeant cation of IP_3R and RyR with an appreciable electrochemical gradient. Despite the weak cation selectivity of both channels (P_{Ca}/P_K ~ 7) (*111, 184*), they are therefore likely to conduct mainly Ca^{2+} when they open within these membranes. The SERCA, by selectively accumulating Ca^{2+} into these organelles, ensures that IP_3R and RyR behave as intracellular Ca^{2+} channels; in effect, the role of selecting ions has been delegated to the SERCA. The situation is very different when IP_3R or RyR find themselves in the PM, where their opening is likely to provide routes for inward fluxes of Ca^{2+} and Na^+ and outward fluxes of K^+. Furthermore, gating of RyR is modulated by membrane potential with steps to hyperpolarized or depolarized potentials of more than ~40 mV causing an initial activation and then inactivation (*191*). The effects of membrane potential on IP_3R are less clear, with suggestions that IP_3R activity is either unaffected (*192, 193*) or weakly enhanced by

depolarization (*194, 195*). Depolarization and hyperpolarization have also been reported to increase γ by relieving the Mg^{2+} block of the channel (*196*). These effects of membrane potential on RyR and perhaps IP_3R have been rather neglected because within the ER/SR such regulation is unlikely to be significant, but it may be very important when IP_3R or RyR are expressed in the PM. Within the PM, therefore, IP_3R and RyR may regulate both membrane potential and Ca^{2+} entry, and their activity may also be regulated by membrane potential.

The behavior of IP_3R (*111, 197, 198*) and RyR (*199, 200*) is modulated by their association with many different accessory proteins. These provide additional levels of regulation of channel gating, directly by the associated proteins (*199, 201, 202*), via phosphorylation (*203, 204*), or via the proteins serving as sensors of, for example, redox potential (*205*) or Ca^{2+} (*206*). Other associated proteins, for example, IRBIT (*207, 208*) and CaM-KII (*17*), are directly regulated by the active channels. These accessory proteins include those, like chromogranin (*209*), ERp44 (*205*), and calsequestrin (*200*), that are expressed within the ER/SR lumen and others, like calnexin (*210*), junctin, and triadin (*200, 211*), that are expressed only in ER/SR membranes. Each of these channels therefore provides a scaffold around which a signaling complex is assembled, which then defines the complex integrative behavior of IP_3R and RyR. The components of these signaling complexes must be different for channels in the ER/SR and PM, not least because the latter are devoid of luminal proteins. As proteins pass through post-ER compartments, attached carbohydrates are further processed, and these modifications may also affect the behavior of IP_3R and RyR that progress to the PM. It has, for example, been suggested that PM IP_3R in lymphocytes are enriched in sialic acid and differ from intracellular IP_3R in their affinity for IP_3 (*129*). The key points are that IP_3R and RyR within the PM may, by assembling into different signaling complexes, differ from their intracellular counterparts both in their regulation and in the downstream proteins to which they signal.

Ca^{2+} channels within the ER/SR access a finite Ca^{2+} store, whereas those within the PM have access to an unlimited pool of extracellular Ca^{2+}. The difference is likely to affect the duration of the Ca^{2+} signals evoked by IP_3R and RyR in the two settings, and the impact of feedback regulation by luminal Ca^{2+} concentration on channel gating (*206, 212*). Finally, because Ca^{2+} signals can be locally decoded, the Ca^{2+} released by channels within the ER/SR is likely to recruit the activity of different Ca^{2+} sensors to those arising from PM channels. Ca^{2+} signals emanating from IP_3R (or RyR) within the PM will, therefore, have different spatiotemporal profiles compared to those arising from the same channels with the ER/SR, and each may thereby regulate different cellular responses.

In the remainder of this final section, we consider the likely physiological significance of these effects of RyR and IP_3R in the PM.

Bone mass in adults is maintained by the counteracting activities of the osteoblasts that deposit bone and the osteoclasts that resorb it. Numerous feedback loops coordinate the activities of these two cell types (*213*), one of which is inhibition of the resorptive activity of osteoclasts by high local extracellular Ca^{2+} concentrations, which trigger an increase in cytosolic Ca^{2+} concentration (*214*). CaR, with its huge extracellular region that binds Ca^{2+} (*176*), mediates the responses of many cells, like osteoblasts (*215*) and parathyroid chief cells (*216*), to changes in extracellular Ca^{2+} concentration. The role of the CaR in

osteoclasts has been more contentious, although osteoclasts express CaR (*217, 218*) and loss of CaR severely compromises their responses to extracellular Ca^{2+} concentration (*218*). These observations are consistent with CaR, via activation of phospholipase C and an increase in cytosolic Ca^{2+} concentration, playing a role in feedback inhibition of osteoclast activity by extracellular Ca^{2+} (*219, 220*). Zaidi and his colleagues have suggested that RyR2 within the PM of osteoclasts may further contribute to these Ca^{2+}-regulated pathways, minimally by providing a route for entry of Ca^{2+} across the PM, but perhaps also by providing an additional sensor for extracellular Ca^{2+} (*221, 222*). The latter suggestion derives from the observation that RyR within the more typical setting, the SR, are regulated by luminal Ca^{2+} (*223*). Several arguments suggest that the luminal surface of RyR2 is unlikely to serve as a sensor of extracellular Ca^{2+} in osteoclasts. First, the affinity of the luminal Ca^{2+}-binding site on RyR2 ($K_D^{Ca} \sim 40\,\mu M$) (*212*) is far too high, even allowing for some competition with extracellular Mg^{2+}, to respond to the changes in the extracellular Ca^{2+} concentration (several millimolar) to which osteoclasts respond. Second, it seems likely though perhaps not proven that accessory proteins, such as luminal calsequestrin (*224*), mediate regulation of RyR2 by luminal Ca^{2+}. Such proteins would be unlikely to associate with the extracellular surface of RyR in the PM. Finally, the contributions from intracellular Ca^{2+} stores and Ca^{2+} entry (*182, 225*) and the pharmacology of the intracellular Ca^{2+} signals evoked in osteoclasts (stimulation by extracellular Ca^{2+}, Cd^{2+}, and Ni^{2+}) (*226*) seem more likely to reflect initiation of the Ca^{2+} signals by CaR (*227, 228*) rather than RyR. It seems very unlikely, therefore, that a PM RyR serves as an extracellular Ca^{2+} sensor in osteoclasts. Indeed, until there is more compelling evidence in support of a functional RyR in the PM of osteoclasts, whether the RyR plays any direct role in mediating Ca^{2+} entry, rather than simply fulfilling its more conventional role as an intracellular Ca^{2+} channel, in osteoclasts remains an open question.

Secretion of insulin from pancreatic β-cells is regulated by the synergistic actions of glucose and gut hormones (incretins) that stimulate cAMP formation. Glucose metabolism causes an increase in cytosolic ATP concentration, which closes K_{ATP} channels, leading, via an unidentified leak channel, to depolarization of the PM and thereby activation of Ca_v channels, an increase in cytosolic Ca^{2+} concentration, and exocytosis of insulin-containing vesicles (*229*). Treatment of type 2 diabetes mellitus with sulfonylureas, which close K_{ATP} channels, affirms the importance of this pathway in insulin secretion, but it is clear that glucose can also stimulate insulin release via pathways, presently ill-defined, that do not require closure of K_{ATP} channels (*230−232*). One of several possibilities (*233*) is that this second pathway requires RyR. Functional RyR, most likely RyR2, are certainly expressed in insulinoma and pancreatic β-cells (*170, 234*), and stimulation of RyR can evoke insulin release (*170, 235, 236*). Whether RyR are required for glucose-evoked insulin release is less clear (*168, 170, 236−242*). There is, however, evidence that development of type 2 diabetes is associated with a loss of functional RyR2 (*170, 243, 244*).

Minimally, RyR within β-cells seem able, via Ca^{2+}-induced Ca^{2+} release, to amplify the Ca^{2+} signals evoked by glucose-stimulated Ca^{2+} entry (*234, 245−247*). Because the sensitivity of RyR to Ca^{2+} is modulated by many additional signals, including cyclic ADP ribose, ATP, cAMP, and redox state, other signaling pathways, the incretins, for example, may regulate the gain on this relationship between Ca^{2+} entry and its amplification by

intracellular Ca^{2+} stores. RyR within the PM might fulfill a similar role by coordinating responses from different signaling pathways and transducing them into opening of a channel that would both depolarize the PM (by allowing Na^+ entry) and provide a direct route for Ca^{2+} entry. Expressing a very high-conductance, nonselective cation channel (the RyR) in the PM of an electrically excitable cell (the β-cell) might seem to be a dangerous undertaking, but it is worth recalling that RyR are also regulated by membrane potential, such that they rapidly inactivate after step changes to either hyperpolarizing or depolarizing potentials (*248*). It may therefore be that within the PM, RyR transiently open only when provided with coincident stimuli: a step change in membrane potential and delivery of an appropriate cytosolic signal, such as Ca^{2+} or cyclic ADP ribose. Similar considerations might be important determinants of RyR activity in cardiac myocytes (*188*, *189*) or vascular smooth muscle (*163*). A further level of control might be imposed by dynamic trafficking of RyR to and from the PM of β-cells. Within insulinoma cells, RyR are expressed within insulin-containing vesicles (*249*) and/or endosomes (*170*), suggesting that both secretion of insulin and the subsequent retrieval of the membrane by endocytosis (*250*) may be intimately associated with regulated expression of RyR in the PM. Further work is required to extend results with insulinoma cells to primary β-cells and so establish whether they too express functional RyR2 in the PM, to determine whether PM expression of RyR2 is dynamically regulated, and to establish the consequences for β-cell physiology of gating RyR within the PM.

The evidence that IP_3R are expressed in the PM of DT40 cells is compelling (*65*, *120*, *140*, *141*, *143*, *251*). Rather less secure, because it rests more on correlative evidence (see above), is our suggestion that IP_3R in the PM are entirely responsible for the BCR-evoked Ca^{2+} entry that occurs via a non-SOCE pathway (Figure 2A). That conclusion implies that the two or three IP_3R found in the PM of each DT40 cell are responsible for approximately half the Ca^{2+} entry evoked by the BCR (Figure 2A) (*120*). Our earlier analysis (*65*), in which we used the measured Ca^{2+} conductance of the PM IP_3R ($\gamma_{Ca} \sim 9$ pS), its open probability when maximally activated by IP_3 ($P_o \sim 0.24$), and the number of IP_3R expressed in the PM (~ 2) to estimate the likely flux of Ca^{2+} ($\sim 4 \times 10^5$ Ca^{2+}/s) through the PM IP_3R, suggested that two IP_3R in the PM are sufficient to mediate the Gd^{3+}-insensitive Ca^{2+} entry evoked by activation of the BCR (Figure 2A). The remaining Ca^{2+} entry, via SOCE, occurs via some 10000 or more Orai channels (*65*, *252*). We speculate, although without specific evidence yet, that similar amounts of Ca^{2+} gushing into the cell via just two or three IP_3R is likely to generate very different local Ca^{2+} signals and regulate a different response to that dribbling into the cell via 10000 low-conductance Orai channels (*16*). The effects of the two pathways on membrane potential are also likely to differ: opening of IP_3R within the PM is likely to cause depolarization, whereas Orai channels are exquisitely Ca^{2+}-selective and unlikely to regulate membrane potential directly. Whether IP_3R in the PM, where they may be closely associated with the signaling machinery that generates IP_3, respond differently to intracellular IP_3R when cells are stimulated is another issue that needs to be resolved.

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