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Calcium leak from intracellular stores—the enigma of calcium signalling

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Summary Wherever you travel through the cytoplasm of the cells you will find organelles with internal [Ca^{2+}] levels higher than in the surrounding cytosol. This is particularly true of the endoplasmic reticulum (ER) (or sarcoplasmic reticulum (SR) in muscle cells); such organelles serve as the main sources of releasable Ca^{2+} for cytosolic cellular signalling. Calcium pumps of the SERCA family (sarcoplasmic and endoplasmic reticulum calcium ATP-ases) import calcium into the organelle lumen. The other mechanism that is responsible for the steady state calcium level within the lumen of ER or SR is a calcium leak that balances the influx created by the pumps. The leak remains the most enigmatic of the processes involved in calcium regulation. The molecular nature of the leak mechanism is not known. The basal leak is a relatively slow process, which is difficult to investigate and which is easily outmatched (both in the amplitude of calcium responses and in attractiveness to experimenters) by substantially faster second messenger-induced release. Nevertheless, information on the properties of the calcium leak, although thinly scattered through the pages of PubMed, has been slowly accumulating. In this review we will discuss the properties of the calcium leak and speculate about possible mechanisms, which could mediate this process.

BASAL CALCIUM LEAK—MEASUREMENTS, PHARMACOLOGY AND KINETICS

An elegant technique of trapping low affinity calcium indicators inside cellular organelles has allowed experimenters to visualise the calcium leak in the ER of individual permeabilised [1,2] (see also Fig. 1A) or patch-clamped cells ([3–5] see also Fig. 1B). The detailed study by Hofer et al. describes the process of calcium leak unmasked by the inhibition of calcium pumps [6]. This process of calcium leak manifests itself as a decrease in free calcium concentration in the ER lumen ($[Ca^{2+}]_{ER}$) following treatment with the SERCA inhibitor thapsigargin (Fig. 1). The leak is neither affected by inhibition of ryanodine receptors with ruthenium red, nor by blocking of IP_3 receptors with heparin [6]. The conclusion that the "basal" calcium leak is not mediated by IP_3 receptors was further supported by studies employing $^{45}Ca^{2+}$ to monitor unidirectional calcium transport from ER [7]. In our experiments in patch-clamped and permeabilised pancreatic acinar cells, inhibitors of ryanodine receptors, IP_3 receptors and of Ca^{2+} release by NAADP were also without effect on the rate of the basal Ca^{2+} leak [8]. Studies of the calcium leak in permeabilised hepatocytes reached similar conclusion. Using measurements of $^{45}Ca^{2+}$ transport, Beecroft and Taylor found that basal calcium leak is not mediated by IP_3 receptors [9]. Ryanodine receptors are also not involved in basal leak in this cell type since high concentrations of ryanodine, ruthenium red or tetracaine had no effect on the calcium content of the stores [10]. In A7r5 cells caffeine, theophylline, xestospongin C and heparin inhibited IP_3-induced calcium release but had no pronounced effect on basal calcium leak [11,12]. Missiaen et al. have systematically investigated the effect of different doses of IP_3 on calcium efflux from the store of permeabilised A7r5 cells. Their data suggest that at concentrations of IP_3 of less than a few tens of nM the basal non-IP_3 mediated leak makes the major contribution to the total calcium efflux [7].

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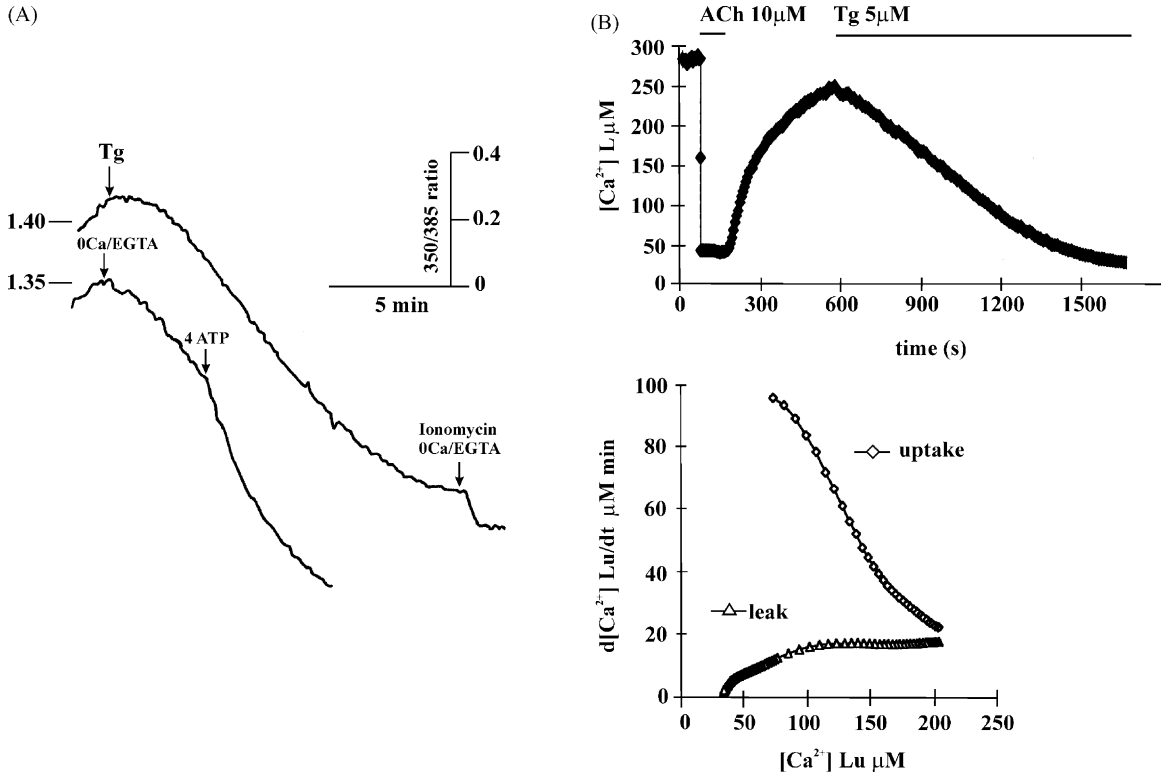

Fig. 1 Examples of recordings of passive calcium leak from the intracellular stores. The intracellular stores of the cells were loaded with the low affinity calcium indicator Mag-fura-2. (A) Recordings of the leak from BHK-21 cells, permeabilised with streptolysin-O. Note the two ways of unmasking the leak: thapsigargin (TG) treatment (upper trace) and lowering of external calcium (lower trace). The lower trace illustrates the accelerating effect of ATP on passive leak. The numbers on the right represent ratios of Mag-fura-2 fluorescence (350/385 nm). The time scale and the scale of proportional Mag-fura-2 ratio changes are shown on the right (reproduced with permission from [6]). (B) Changes of calcium concentration in the ER of patch-clamped (whole-cell configuration) pancreatic acinar cells. The patch pipette solution was supplemented with BAPTA/Ca buffer. The upper part of the figure allows the comparison of calcium changes in ER induced by the application of the calcium-releasing agonist acetylcholine (ACh), recovery of the store calcium due to activity of calcium pumps and finally depletion due to passive leak. The lower part of the figure shows the dependence of calcium leak and calcium uptake on intra-ER calcium (reproduced with permission from [5]).

It seems, therefore, that the basal leak from the ER is not mediated by any of the known classes of calcium-releasing channels.

There are however some substances which have been found to affect the leak rate. Let us summarise this "positive" pharmacology of the leak. Calcium leak was found to be stimulated by ATP in some [6], but not all [13] cell types. In experiments on permeabilised BHK-21 cells the increase of ATP concentration from 0.375 to 4 mM drastically accelerated the basal calcium leak [6] (Fig. 1A). Glutathione, a tripeptide which is an important reducing agent in the cytosol, can accelerate the basal leak; both the oxidised and reduced forms of glutathione are capable of activation but the reduced form is more effective [14]. The basal leak has recently been shown to be inhibited by Ni^{2+}, which is also known to block some types of plasma membrane calcium channels [10]. This sensitivity plus the relatively shallow temperature-dependency of the basal leak [9,15] suggest that the leak is mediated by a specialised calcium channel [10]. However, the evidence in favour of a channel is indirect. The dependency of the leak on intra-ER calcium concentration revealed an interesting mechanism for its regulation. Measurements of the calcium dependency of the leak in patched-clamped pancreatic acinar cells (see Fig. 1B), revealed an almost constant leak rate in a broad range of calcium concentrations and very low intensity of the calcium efflux at luminal calcium levels below 40 μM [5] (Fig. 1B). A number of other studies have indicated that the leak drastically slows down when luminal $[Ca^{2+}]$ levels decline to some tens of micromoles per litre—concentrations that are still far from equilibrium with the cytosolic concentrations [6,8,16] (Fig. 1). Measurements using $^{45}Ca^{2+}$ also indicated the drastic decrease of leak rate at the store calcium levels above complete depletion [9]. This effect could be potentially attributed to the voltage difference between the ER and cytosol. There is undeservedly little information on voltage gradients between cellular organelles and

cytosol (with the exception of mitochondria). We know, however, that efficient charge compensation mechanisms are developed in releasable calcium stores to compensate for much faster calcium fluxes than the efflux generated by calcium leak [17,18]. Furthermore, there are no indications of accumulation of voltage-sensitive indicators in the ER therefore the existence of large voltage gradients between the ER and cytosol is unlikely. Another possible explanation for the undepletable calcium plateau is that luminal calcium might enzymatically modify the leak channels, preventing a complete depletion of ER calcium. Proof of this hypothesis would require the identification of putative molecules responsible for the inhibition of the leak channels at low intrastore calcium levels (or the opening of these otherwise closed channels, at higher calcium levels), or demonstration that calcium ions acting from the lumen of the store could directly activate the leak channel. Finally, one cannot exclude the existence of another calcium store which could accumulate calcium indicator or $^{45}Ca^{2+}$, but which possesses a much slower leak pathway and/or a much lower sensitivity to inhibitors of SERCA pumps than the main thapsigargin-depletable store. Indeed calcium leak from the Golgi apparatus, an organelle which has been shown to possess properties of a messenger-releasable calcium store [19] displays a much lower sensitivity to thapsigargin and substantially slower calcium leak than the ER [20,21]. The existence of such a separate, undepletable store could provide an alternative explanation for the observed effect. Although sharp decreases of calcium leak and consequent formation of plateau levels of undepletable calcium in the calcium stores have been found in a number of studies, the mechanism of this phenomenon is not entirely clear and could be different in different cell types.

The kinetics of calcium leak at higher intraluminal calcium concentrations is uneventfully linear (Fig 1B) [5,16] or monoexponential [9]. Linearity indicates saturation of the leak channels with calcium whilst monoexponential decline represents simple proportional calcium dependency. More complex kinetics of two-exponential decline of intrastore calcium was also reported [7].

HOW FAST IS THE BASAL LEAK FROM ER?

From studies using free calcium measurements in internal stores, one can find values for the decrease of $[Ca^{2+}]$ in the stores per unit time ($\Delta[Ca^{2+}]_{ER}/\Delta t$)—usually per minute. The majority of such measurements were performed at room temperature (20–25 °C). The maximal leak rate, induced by inhibition of calcium pumps, was approximately 19 μM/min in pancreatic acinar cells [5]. Similar values of leak of a few tens of μM per minute could be derived from studies of BHK-21 cells [6,22]. A higher intensity of leak—approximately 90 μM/min was found in sensory neurons [16]. Estimations made from data published by Barrero et al. [15] gives even higher values of leak from ER of HeLa cells 100–200 μM/min. The rate of leak in terms of proportional changes of total releasable calcium could be found in studies that utilised $^{45}Ca^{2+}$ measurements. In A7r5 cells, inhibition of calcium pumps by thapsigargin resulted in a maximal fractional calcium content loss of 22% per minute [7]. Similar values of approximately 15% per minute can be derived from experiments on COS-1 cells reported by the same group [20]. In hepatocytes the leak-induced calcium loss also seems to occur with the rate of a few tens of percents per minute. In permeabilised thapsigargin-treated hepatocytes, monoexponential decline of calcium in the stores with $\tau_{1/2}$ of 208 s at 20 °C [9] was reported; $\tau_{1/2}$ decreased to 111 s at 37 °C. The pancreatic acinar cell is one of a very few cell types for which the strength of calcium buffering in the ER is known—the calcium binding capacity of ER was estimated to be approximately 20 [23]. This allows the possibility of estimating the rate of decrease of total calcium concentration in ER lumen ($\Delta[Ca]_{ER}/\Delta t$) due to leak—the calculated value is approximately 400 μM/min.

In the majority of cell types studied, the passive leak is capable of completely depleting the calcium stores in a few minutes.

PAYMENT FOR IMPERFECTION OR LEAK THROUGH THE PUMPS AND TRANSLOCONS

The process of calcium leak is frequently studied by monitoring intra-ER calcium content after application of SERCA pump inhibitors—these conditions potentially eliminate the calcium leak component due to the reverse calcium flux through the pumps. Another way of unmasking the leak is by reducing the "cytosolic" calcium to levels that cannot activate the pump (substantially below the resting cytosolic level—Fig. 1A). In this situation the forward, but not reverse, mode of the pump is inhibited. Another way of eliminating calcium uptake is by removal of ATP (usually accompanied by addition of Apyrase). Such protocols of leak measurements allow the resolution and comparison of pump-dependent and -independent components. The contribution of these components is clearly different in different cell types. In SR vesicles isolated from skeletal muscles, thapsigargin and CPA have a drastic effect on total calcium leak induced by removal of ATP from the media [24], suggesting that the reverse mode of the pumps is the main contributor of the passive leak in this cell type. Interestingly, structural properties of SR pumps indicate that they should mediate considerable leakage and that this leakage should be sensitive to thapsigargin [25]. The conclusion about the important contribution of calcium "back-flux" through the pumps to the total SR calcium leak was also reached in other studies on striated

muscles [26,27]. However, in other cell types, inhibition of the pumps with thapsigargin had no pronounced effect on the leak from calcium stores [6,9], suggesting that the main leak component is not mediated by calcium pumps (Fig. 1A). One possible explanation for differences in the behaviour of calcium stores is that different types of SERCA pumps are expressed in these stores and these pumps could possess different leakiness. An alternative explanation is that in the highly specialised SR, where calcium pumps are by far the most abundant proteins on the surface of the organelle, the leak is determined by intrinsic leakiness of the pumps; in contrast, on the ER membranes of other cells where the pumps are less abundant, different mechanisms have evolved to mediate the leak.

In pancreatic acinar cells the basal leak was stimulated by puromycin—an antibiotic that purges translocons from nascent polypeptide chains [8]. Translocons are protein conducting channels that can be found on the surface of rough ER. The experiments with puromycin suggest that a component of basal leak could be mediated by translocons. Secretory cells that specialise in the production of proteins for export have highly developed rough ER. The pancreatic acinar cell is an extreme example of such secretory machinery with rough ER occupying more than 20% of cell volume—indeed the basal part of the cell is packed with endoplasmic reticulum and studded with ribosomes [28]. The well-developed rough ER is the main source of releasable calcium in pancreatic acinar cells. The electrophysiological studies in the late 1980s and early 1990s demonstrated that puromycin induces the opening of large conductance translocon channels [29,30]. Even more importantly, spontaneous opening of translocon channels was also reported [29] and could mediate calcium leak in pancreatic acinar cells and other cell types. In spite of the vast number of ribosome–translocon complexes the calcium leak even in pancreatic acinar cells is relatively small (less than 25% of maximal flux of calcium pumps). When ribosome–translocon complexes are free from polypeptide chains they conduct both ions and neutral molecules [30,31]. High permeability of translocon channels to neutral molecules suggests that calcium could leak from the ER if it forms neutral complexes with small molecules; this possible leak mechanism has not been tested experimentally. Nature clearly attempted to create the protein conducting channels in a calcium-tight way. Indeed the ribosome–translocon complex does not conduct ions when it is occupied by growing polypeptide chain [30]. In the alternative configuration, when the ribosome is detached from the complex, the translocon channel is blocked by ER luminal protein BiP [32]. The slow basal leak, which is evident in secretory cells, could be simply a consequence of the imperfections of the design of the protein translocation system. It is important to note that some ribosomes continue to associate with translocons even after the termination of protein syntheses [33]; these complexes could play particularly important roles in the mediation of leak. Interestingly in COS-1 cells the basal calcium leak from the Golgi apparatus (which does not contain ribosome–translocon complexes) is substantially slower than the leak from the ER [20]. Indeed, if the translocons are important mediators of the leak then non-ER organelles (e.g. Golgi, secretory vesicles) might be constructed to have a substantially slower calcium leak.

Not all translocon-free organelles have a low calcium leak rate. Unlike Golgi, endosomes are apparently quite leaky. When endosomes are formed they have a calcium concentration equal to that of extracellular solution (in the millimolar range). However, already in early endosomes, free calcium has fallen to micromolar levels [34]. Calcium uptake into early endosomes, followed by calcium leak could constitute a calcium influx into the cytosol, that could be considerable in some cell types [34].

BCL-2 AS A LEAK AND SURVIVAL MECHANISM

Let us return to the main intracellular calcium store—the endoplasmic reticulum. Using ER–targeted aequorin, Pinton et al. from Rizzuto's laboratory demonstrated that overexpression of the small anti-apoptotic protein Bcl-2 decreases the calcium content of the ER. The effect was proven to be due to an increase in calcium leak [35]. This study also reports similar effect of Bcl-2 overexpression on Golgi calcium [35], suggesting that Bcl-2 is a candidate for the role of the universal mediator of organellar calcium leak. The effect of Bcl-2 on calcium leak from ER was soon confirmed in experiments employing a targeted fluorescent cameleon [36]. In these experiments, cells in which Bcl-2 was overexpressed showed approximately twice the rate of ER calcium leak than cells of the wild type [36]. Further studies have linked the increase of ER calcium leak by Bcl-2 with the anti-apoptotic action of this protein [37]. A number of other effects of Bcl-2 on calcium homeostasis have been also reported [38,39]. An overload of the calcium stores is as dangerous to cells as an overflow of the mountain lake to dwellings of nearby valleys. The amplitude of intra-ER calcium needs to be carefully managed, otherwise the useful calcium irrigation of cytosolic responses could be easily converted to a destructive calcium flood. Different ways of decreasing intra-ER calcium (PMCA overexpression, tBuBHQ treatment) have been shown to limit cell susceptibility to noxious stimuli [37]. The direct demonstration of the importance of calcium leak (Bcl-2 mediated leak in these experiments) in limiting cellular damage emphasizes an important function of the leak pathway in preventing ER calcium overload and generally in maintaining safe cytosolic calcium signalling [37].

BASAL LEAK AND PHYSIOLOGICAL LEAK

The analytical measurements of basal calcium leak are usually performed on permeabilised or whole-cell patched cells. The basal leak measured in such experiments is probably different from the "physiological" calcium leak. There are two main reasons for such difference. The first reason is the removal of possible endogenous cytosolic activators such as basal levels of IP_3, cADP ribose, NAADP, glutathione and potential inhibitors of calcium leak during permeabilisation of the plasma membrane (or during dialysis of the cytosol via the patch pipette); this, however, is almost unavoidable in actual experimental conditions. The second reason is that whilst in their natural environment (in tissues of the body) cells even at "rest" are always exposed to some level of calcium-releasing hormones and neurotransmitters whilst in experimental conditions unstimulated cells are free from such influences. Recently in pancreatic acinar cells we attempted to compare the basal leak with the "minimal physiological" leak [8]. CCK is an important calcium-releasing hormone which regulates pancreatic secretion. One picomole of CCK (the concentration seen in plasma in the fasting state) resulted in small but measurable changes of the intra-ER calcium level [8]. The effect of this concentration of CCK on the rate of calcium leak, unmasked by inhibition of pumps with CPA, was more difficult to resolve. The acceleration of the CPA-induced leak by 1 pM CCK was not statistically significant [8]. These experiments suggest that the basal leak is the main contributor to the minimal physiological leak in this cell type. In the same study 10 pM CCK, a concentration seen in plasma after a meal, increased the rate of CPA-induced calcium efflux by more than 2.5 times [8].

In physiological conditions "natural" ionophores could also contribute to the calcium leak from internal stores, for example, bile acids have been shown to possess calcium iontophoretic activity, which correlated with hydrophobicity of bile acids [40]. In healthy fasting humans, bile acids are present in serum at micromolar concentrations—the concentration of bile increases to tens of micromoles in certain pathological conditions [41]. In addition to effects on passive calcium transport, bile acids have also been shown to stimulate calcium release from the stores [42–44] therefore these substances could affect ER calcium content by two separate mechanisms.

In excitable cells and in neurons in particular, intracellular stores undergo calcium overcharge after periods of electrical activity. The opening of voltage-gated calcium channels increases calcium in the cytosol and shifts the balance between the leak and uptake, in favour of uptake. The stores accumulate additional calcium, meanwhile cytosolic calcium is rapidly cleared by sodium–calcium exchangers and calcium pumps. From that moment the calcium concentration in the stores is higher than the equilibrium level with regards to cytosolic calcium—the stores are overcharged. The kinetics of recovery from calcium overcharge have been measured by Garaschuk and Yaari from Konnerth laboratory [45]. In their experiments the recovery from overcharge took a considerable time (*6–9* min) [45]. Following calcium release in non-excitable cells the formation of a calcium steady state in the ER starts by net calcium uptake (and leak slows down the process) whilst in excitable cells the restoration of a calcium equilibrium in stores, overcharged by the opening of voltage-gated channels, depends on the net calcium leak and the uptake opposes this process. It is not clear whether resting leak and recovery from overcharge are mediated by the same mechanism.

For some cell types the very notions of "resting" and "physiological" calcium leak are not applicable. These cells do not form long-standing calcium levels inside stores

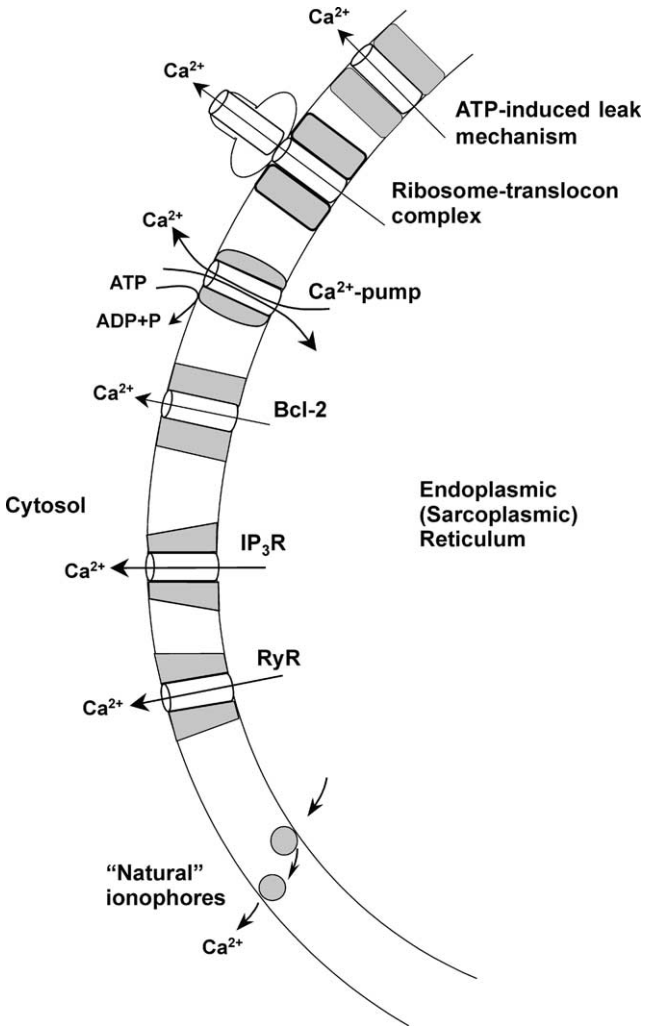

Fig. 2 Possible mediators of the leak from the endoplasmic (sarcoplasmic) reticulum.

based on the equilibrium of uptake by pumps and efflux by calcium leak mechanism. These cells do not maintain sustained calcium levels in preparation for hormonal stimulation; in fact they continuously undergo powerful calcium releases from stores followed by fast recoveries. The cardiomyocyte, which in physiological conditions rhythmically undergoes CICR, is a good example of such a cell. The calcium efflux during CICR is probably much faster than that attainable by passive leak. The dynamic control of SR calcium levels is achieved mainly by regulating efficiency of CICR and of uptake into SR [46,47]. Because of continuously changing intrastore calcium levels the passive component of the efflux must be also changeable and its contribution to total efflux difficult to determine.

CONCLUSIONS

Fig. 2 summarises possible mechanisms that could mediate calcium leak; at the moment, however, this drawing is a fantasy map of Terra Incognitae. Finding out the exact mechanisms that mediate both "basal" and "physiological" calcium leak remains a challenge to scientists working in the calcium signalling field. In every cell's calcium signalling toolkit (a term coined by Berridge, Lipp and Bootman [48]) one would be able to find a calcium leak–not flashy but a useful instrument. In spite of its slow rate the leak determines starting calcium concentrations in internal stores and because of that, it is one of the essential mechanisms, involved in formation of cytosolic calcium signals. Essential yet unknown–these two properties of the leak are excellent justifications for further studies of this mechanism.

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