First publ. in: Cell Calcium 38 (2005), pp. 319-327

Molecular aspects of rapid, reversible, Ca^{2+}-dependent de-phosphorylation of pp63/parafusin during stimulated exo-endocytosis in *Paramecium* cells

Helmut Plattner*, Roland Kissmehl

Department of Biology, University of Konstanz, P.O. Box 5560, 78457 Konstanz, Germany

Received 20 June 2005; accepted 28 June 2005
Available online 15 August 2005

Abstract

Ca^{2+} signalling governs stimulated exocytosis and exocytosis-coupled endocytosis also in *Paramecium* cells. Upon stimulation, the $\leq 10^3$ dense-core exocytotic organelles (trichocysts) can be synchronously (80 ms) released, followed by endocytotic membrane resealing (350 ms) and retrieval. *Paramecium* is the most synchronous dense-core exocytotic system known, allowing to dissect rapidly reversible Ca^{2+}-dependent phenomena. This holds for the reversible de-/re-phosphorylation cycle of a 63 kD phosphoprotein, pp63/parafusin (pf), which we have cloned, immuno-localised, and characterised as phosphoglucomutase, the enzyme funneling glucose into the glycolytic pathway. It was isolated ex vivo, followed by MALDI analysis, while X-ray structure analysis was performed after heterologous expression. We found multiple phosphorylation of superficial Ser/Thr residues. Although present also in exo^- mutants, pp63/pf is selectively de-phosphorylated only in exo^+ strains during synchronous exocytosis (80 ms) and re-phosphorylated within ~20 s, i.e., the time required to re-establish $[Ca^{2+}]$ homeostasis. We have isolated relevant protein phosphatases and kinases and probed their activity on pp63/pf in vitro. We consider Ca^{2+}/calmodulin-activated PP2B (calcineurin, whose subunits have been cloned) relevant for de-phosphorylation. Re-phosphorylation can be achieved by two protein kinases that also have been cloned. One is activated by cGMP (PKG) which in turn is formed by Ca^{2+}-activated guanylate cyclase. Another kinase, casein kinase 2, is inhibited by Ca^{2+} and, hence, activated with some delay in parallel to decreasing $[Ca^{2+}]$ after exocytosis. In total, several Ca^{2+}-sensitive cycles cooperate whose protein components have been localised to the cell cortex. Regulation of the phosphorylation degree of pp63/pf may affect structure binding on a microscale and/or its enzymatic activity. All this may serve fueling substrate into glycolysis with increased ATP re-formation (compromised in exo^- mutants) and NADH formation, with effects on Ca^{2+} signalling including mobilisation from cortical stores (alveolar sacs) and overall effects on ATP and Ca^{2+} dynamics during synchronous exo- and endocytosis.

Keywords: Ca^{2+}; Calcium; Exocytosis; Parafusin; *Paramecium*; Phosphorylation

1. Introduction

In eukaryotic cells, Ca^{2+} governs exocytosis and endocytosis, as well as intracellular trafficking [1,2]. A swift increase in intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$, occurs in the cell cortex upon exocytosis stimulation [3,4]. All this also holds for the ciliated protozoan, *Paramecium tetraurelia* [5,6]. The ongoing *Paramecium* genome project [7–9] helps to identify molecules involved [10].

* Corresponding author. Tel.: +49 7531 88 2228; fax: +49 7531 88 2245.
E-mail address: helmut.plattner@uni-konstanz.de (H. Plattner).

2. Ca^{2+} signalling in *Paramecium* cells

A *P. tetraurelia* cell contains up to $\sim 10^3$ specialised dense-core secretory organelles (trichocysts) docked at the cell membrane for immediate release upon stimulation, e.g., with the polyamine secretagogue, aminoethyldextran, AED [5,11]. Quenched-flow/cryofixation and freeze-fracture analysis reveals synchronous exocytosis within 80 ms. Together with endocytosis of empty membrane "ghosts" the whole cycle is accomplished within 350 ms [11,12]. Hence, when compared with any other dense-core secretory vesicle system [13], this is the fastest and most synchronous exocytosis sys-

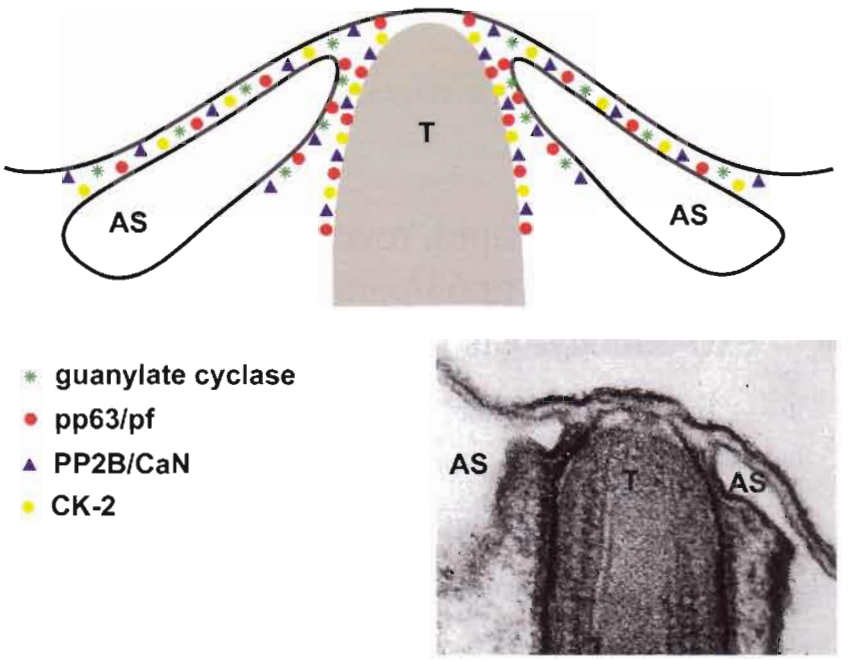

Fig. 1. Trichocyst exocytosis site in *Paramecium*, with the narrow spaces between cell membrane, trichocyst (T) and alveolar sacs (AS), shown as an electron micrograph (magnification 100,000×) and a scheme with the approximate localisation of pp63/pf, PP2B/CaN, CK-2, guanylate cyclase and PKG susperimposed, based on quantitative immuno-gold EM-analysis. For any more details, see text.

tem known [12,14]. The alveolar sacs, cortical Ca^{2+} stores [15,16], are involved [17]. These are the equivalent of the "inner membrane complex" in related pathogenic species, such as *Plasmodium* and *Toxoplasma* for which, however, the store actually relevant for Ca^{2+} signalling during exocytosis-mediated host cell penetration is not known [18]. In *Paramecium*, there is only a ~15 nm wide subplasmalemmal space between the cell membrane and cortical stores that surround trichocyst docking sites (Fig. 1). Their activation is tightly coupled to a SOC mechanism, i.e., a store-operated Ca^{2+}-influx [17,19–21]. This joint activity accelerates all steps of the exo-endocytosis cycle [22]. The Ca^{2+}-release channels of alveolar sacs share important physiological characteristics with ryanodine receptors, RyRs [20] although identification of its gene has not yet been achieved in full. According to fluorochrome analyses, cortical $[Ca^{2+}]_i$ rises with $t_{1/2} \sim 0.3$ s to ~900 nM and, after a plateau of 1.00 s, decays with $t_{1/2} \sim 1.9$ s, to nearly resting levels within ~10 s [20]. Injected Ca^{2+}-chelators indicate requirement of a local $[Ca^{2+}]_i \sim 5\ \mu M$ for exocytosis to occur [23]. Remarkably, not only wildtype cells, but also trichocyst-free (*trichless*, *tl*) and "*non-discharge*" (*nd*) exo^- mutants display Ca^{2+}-signalling when exposed to AED [20,23].

3. De-/re-phosphorylation of pp63/parafusin during stimulated exo-endocytosis

When cells were exposed to the permeabilising fixative, picric acid, pp63/parafusin (pf) molecules were seen to become de-phosphorylated [24,25]. This procedure is routinely used to differentiate between exo^+ and exo^- strains. The real time course can be followed only by stimulation with the secretagogue, AED [26]. For more precision, this has been combined with quenched-flow analysis [12] which revealed that pp63/pf is de-phosphorylated within 80 ms [27]. Although occurring in all strains analysed, pp63/pf is de-phosphorylated only in exo^+ strains [24,26] (Table 1), and re-phosphorylated roughly within ≤20 s [26,28]. Thus, de-phosphorylation coincides with synchronous exocytosis performance [12], as well as with the rise time of cortical $[Ca^{2+}]_i$, as determined by fast confocal fluorochrome microscopy [20] and by electrophysiological recording of Ca^{2+}-activated currents ($t_{1/2} = 21$ ms) measured over the cell membrane during

Table 1
Changes in [ATP] in the different *P. tetraurelia* strains, exo^+ and exo^-, in parallel to pp63/pf de-/re-phosphorylation, analysed during AED-stimulated synchronous exocytosis

Strain	Percentage de-phosphorylation of pp63/pf	Time (s) required for ATP regeneration	
		50% recovery	100% recovery
exo^+ strains			
K401	−66	12.0 ± 1.5	~30
7S	−54	10.5 ± 1.5	~30
d4-500r	−50	11.5 ± 4.0	~30
exo^- strains			
nd9-28 °C	+3	28.5 ± 0.5	>60
nd6	0	58.0	≫60
tl	0	28.5 ± 8.0	>60

Data compiled by [37].

AED stimulation [29]. The much slower re-phosphorylation process roughly parallels re-establishment of $[Ca^{2+}]_i$ homeostasis.

4. Why "parafusin"?

The coincidence of exocytosis competence and pp63/pf de-phosphorylation led to the proposal that this protein may govern membrane fusion, from which the name was derived [30–32]. Cloning of its homologue in *Tetrahymena* and disruption of its single pp63/pf gene suggested this not to be the case [33]. This, however, does not preclude a role in some other phenomena accompanying exocytosis, assuming that a *Paramecium* cell does not de- and re-phosphorylate an important protein without any need. In fact, a role for pp63/pf in *Toxoplasma gondii* is reported in host cell penetration [34]. In sum, its role remained open and will be discussed here in the context of present knowledge.

5. Cloning of the pp63/pf genes, heterologous expression, and immuno-localisation

Starting from an original report [32], we have completed and corrected the published gene sequence and we have found an additional, very similar gene [35]. For heterologous expression in *E. coli*, the deviant codons of *Paramecium* have been mutated to the universal code. Biochemically pp63/pf has been identified as phosphoglucomutase (PGM) [28,35], the enzyme that shuttles glucose into the glycolytic pathway. Both isoforms, differing by only seven aminoacids, possess PGM activity. The properties of the protein extracted ex vivo [28] and of the recombinant form [35] are identical. We also raised polyclonal antibodies (ABs). Immuno-gold electron microscopy (EM) revealed considerable enrichment of pp63/pf in the cell cortex, i.e., at preformed exocytosis sites and around cortical Ca^{2+}-stores, including the narrow subplasmalemmal space [36]. See Fig. 1.

6. MALDI and X-ray structure analysis

The heterologously expressed isoform, pp63/pf-1, has been crystallised and analysed by X-ray diffraction in combination with molecular modelling [37]. The latter was facilitated by the striking similarity with mammalian PGM. Another approach was isolation ex vivo under conditions maintaining phosphorylation, and subsequent peptide mass spectrometric analysis, MALDI [38]. See Fig. 2. This allowed us to identify the following Ser/Thr phosphorylation sites, i.e., S196, T205, T280, T371, T373, and T469, in addition to the enzymatically active site, S126. These sites are prognosticated as being the targets of at least two different types of protein kinases, a casein kinase type 2 (CK-2), and a cGMP-activated protein kinase, PKG. When superim-

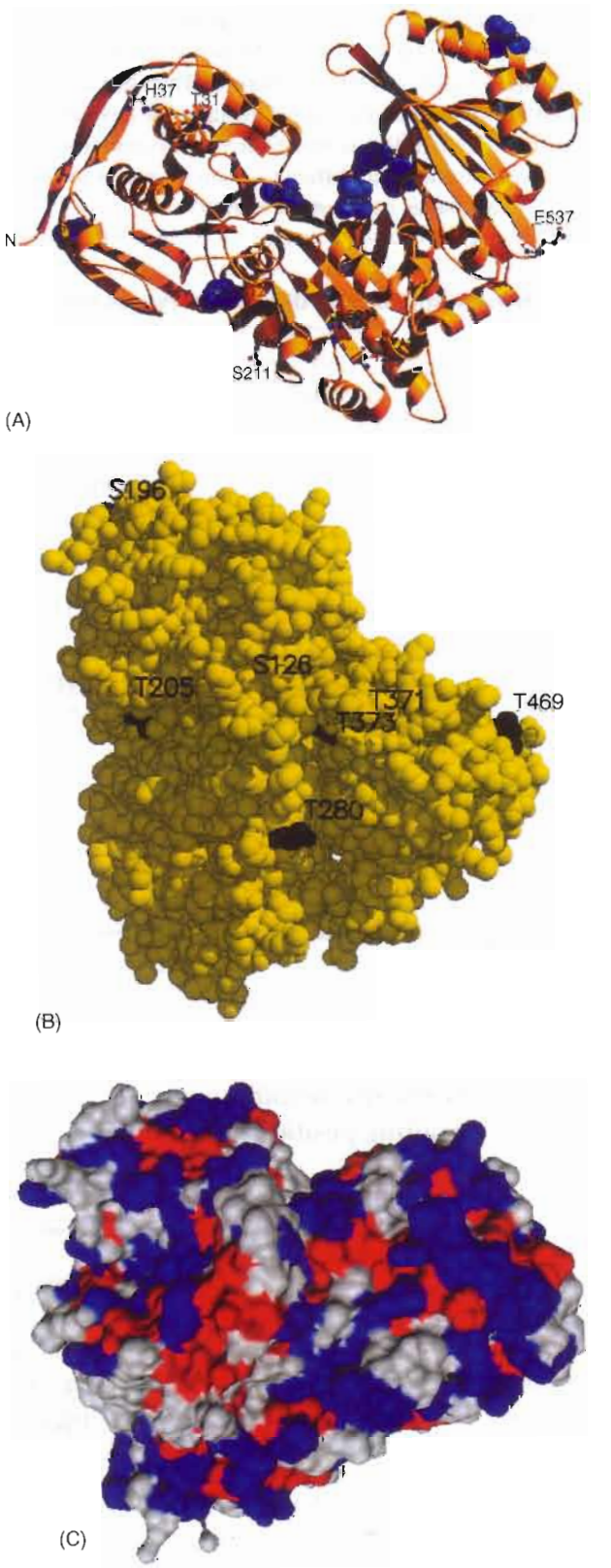

Fig. 2. Molecular structure of pp63/pf from *Paramecium*, with superimposed phosphorylation sites in the ribbon (A) and the space filling model (B). (C) Distribution of hydrophobic (red) as well as of charged (blue) surface details in the recombinant dephospho-form. From [37] and Diploma work by S. Müller, 2000, University of Konstanz.

posed to the X-ray structure, phosphorylation residues are distributed over the surface of the pp63/pf molecule, thus rendering it highly negatively charged. This is in contrast to its non-phosphorylated form and explains the deviation from the estimated overall p*I* derived from the primary structure. In fact, several forms with different p*I* are found on isoelectric focussing gels after isolation ex vivo [27].

7. Protein phosphatase 2B de-phosphorylates pp63/pf

PP2B (calcineurin, CaN), a protein Ser/Thr phosphatase activated by Ca^{2+} and calmodulin (CaM) [39,40], offered itself as a candidate for pp63/pf de-phosphorylation. Both, CaM [41] and CaN [42] are abundant in the *Paramecium* cortex. Injection of anti-CaN ABs, derived from bovine brain, inhibit exocytosis [43]. A protein with considerable resemblance to mammalian CaN has been isolated, biochemically characterised, and shown to de-phosphorylate pp63/pf [44,45]. Its occurrence in *Paramecium* has been consolidated by cloning the subunits (SU). This has been achieved with the catalytic CaM-binding subunit SU-A (unpublished data from our own lab and from the lab of R.D. Hinrichsen [46]) and with the regulatory Ca^{2+}-binding SU-B (our unpublished data). Two forms have been found for each of the subunits. The respective EMBL accession numbers are AF014922 (Hinrichsen lab) and AJ567906 (this lab) for SU-A, as well as AJ554047 and AJ554048 for SU-B, respectively. Immuno-

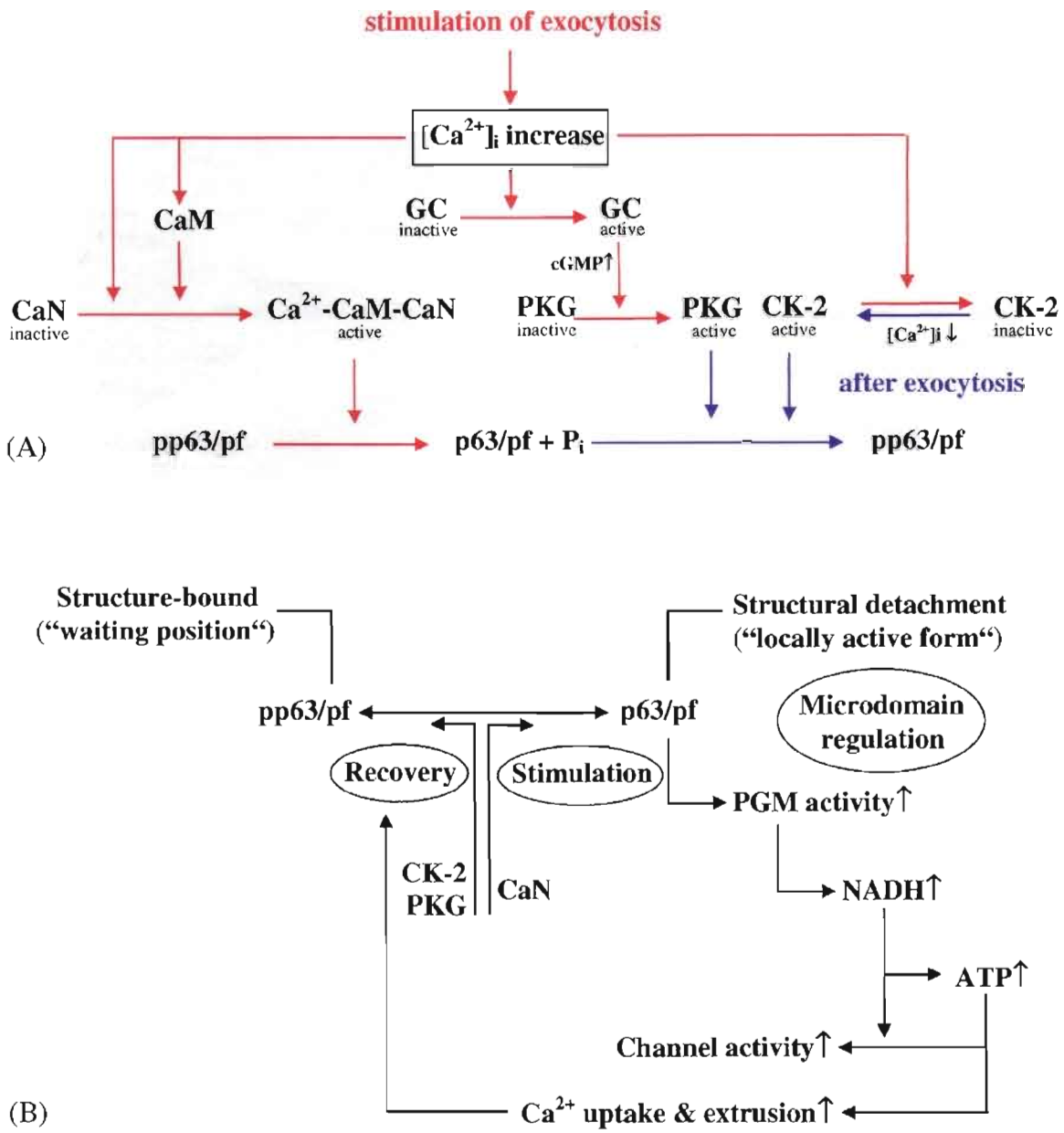

Fig. 3. Flow-chart of interactions proposed in the text, (A) for biochemical steps, and (B) for derived functional steps. GC: guanylate cyclase, P_i: inorganic phosphate. (↑, ↓) Indicate increase and decrease, respectively. For more details, see text.

localisation studies at the EM level showed that both SUs occur in domains predetermined for exocytosis performance and in adjacent areas, i.e., in part in association with alveolar sacs [42]; Fig. 1. Beyond its effect on pp63/pf (Fig. 3A), some other action of CaN cannot be excluded since CaN is also one of the regulators of RyR activity in other systems [47].

8. Possible functional consequences of pp63/pf/PGM de-phosphorylation

Theoretically these findings could imply the following (Fig. 3B). (i) PGM activity may depend on the phosphorylation state. This may be caused not only by phosphorylation close to the active site of the enzyme molecule, but also by an allosteric effect exerted by some more remote phosphorylation sites [37]. (ii) It is known that, depending on the phosphorylation state, proteins may change their association with structural elements, i.e., with membrane lipids or with other proteins [48]. In fact, extensive isolation of pp63/pf/PGM required repeated freezing and thawing, normally indicative of sequestration inside membraneous compartments [27,28]. Upon de-phosphorylation, the molecule may be detached from membranes, while still being retained in the narrow subplasmalemmal space. Abolition of structure-binding may be favourable to substrate turnover, i.e., for accelerating the glycolytic pathway, as has been shown for glycolytic enzymes [49,50] whose proper location is functionally crucial [51].

The arguments along these lines are as follows. In *Paramecium* we have functional evidence for the occurrence of RyR-type Ca^{2+}-release channels in that part of alveolar sacs membranes that face the preformed exocytosis site [6,20]. We currently analyse whether activation entails formation of second messengers derived from NAD(P), nicotinamide dinucleotide (phosphate). Such messengers include the physiological activators of RyR-type Ca^{2+}-release channels, i.e., cyclic adensosine diphosphoribose, cADPR [52,53], and nicotinic acid adenine dinucleotide phosphate, NAADP [54,55] whose synthesis from nicotinamide- and nicotinate-mononucleotide, respectively, requires ATP. For reasons indicated above, ATP regeneration may depend on the phosphorylation state of pp63/pf/PGM—at least a clear correlation occurs (Table 1). If so, it is reasonable to assume that enzymatic turnover, with formation of NADH and ATP, may depend on the frequency of enzyme-substrate interactions. These in turn may depend on mobility in the small subplasmalemmal space, i.e., desorption from structural components by a phosphorylation-dependent change of surface charge of the molecule. In other cells, stimulation entailing cADPR formation is paralleled by a decrease in NAD^+ fluorescence that can be recorded in the entire cell body [52]. If so, this should be even more pronounced within a narrow subplasmalemmal space.

In the *Paramecium* cell cortex, any Ca^{2+}-release channels would have to be located on the outer part of alveolar sacs, because the part facing the cell center is densely studded with Ca^{2+}-pump molecules [56]. In agreement with this postulate, the subplasmalemmal space, including that delineated by alveolar sacs and trichocysts, is the site where pp63/pf [36], as well as the relevant protein phosphatase PP2B/CaN [42] and protein kinases CK-2 and PKG [57,58] are heavily enriched according to immuno-EM studies (see Fig. 1 and below). For an appreciation of the importance of the NAD^+/NADH system for signal transduction (a hypothetic possibility in *Paramecium*), one also has to consider that the RyR molecule contains an oxidoreductase-like domain that is sensitive to these intermediates [59].

9. A Ca^{2+}-inhibitable casein kinase type 2

One of the protein kinases able to phosphorylate pp63/pf has been identified and characterised as casein kinase type 2 (CK-2). This has been achieved after isolation ex vivo [44,60], as well as by cloning of the respective gene(s), specifically of the catalytic SU [57]. This SU has been heterologously expressed and functionally characterised as follows. Quite unusually, kinase activity is inhibited with increasing $[Ca^{2+}]$ (Fig. 3A), while it becomes maximal after Ca^{2+} chelation; $[Ca^{2+}] = 10\ \mu M$ suffices for substantial inhibition. This also holds for the complex formed by the recombinant catalytic SU after reconstitution with the recombinant regulatory SU from *Xenopus laevis* [57]. (This was used in the absence of any information on this SU in *Paramecium* at this time.) Since the magnitude of $[Ca^{2+}]$ that inhibits CK-2 activity corresponds roughly to that locally achieved in vivo during AED stimulated exocytosis [23], this can explain why pp63/pf can be fully re-phosphorylated in vivo only with some delay. Re-phosphorylation takes place as cortical $[Ca^{2+}]_i$ decreases, i.e., with a level-off within 10–20 s [20,23,26,28]. Remarkably, CK-2 is co-localised with pp63/pf/PGM and CaN (Fig. 1).

10. A Ca^{2+}-activated guanylate cyclase and a cGMP-activated protein kinase

During exocytosis stimulation we observe an increase of cGMP [61]. Evidently a guanylate cyclase has been activated—a process known in *Paramecium* to go strictly in parallel to any $[Ca^{2+}]_i$ increase [62]. Cloning and immunogold EM localisation revealed that guanylate cyclase is associated with the (ciliary and non-ciliary) cell membrane and the alveolar sacs membrane complex [63]. Interestingly this is also the distribution of the cGMP-activated protein kinase, PKG [58] (Fig. 1). This enzyme has been isolated ex vivo [44] and it has been shown to phosphorylate pp63/pf [58].

Finally, cGMP is still known to exert some other effects. In some smooth muscle cells, cGMP can directly activate Ca^{2+}-release, i.e., without interaction via a PKG [64]. In many other cells, cGMP can stimulate formation of cADPR [65]. Any details along these lines remain to be elucidated in *Parame-*

cium cells. To be relevant for this aspect, cGMP would have to rise early on during AED stimulation. In reality, cGMP rises only with a delay of ~0.5 s according to quenched-flow experiments [61]. This, however, holds for the overall concentration in the intact cell, while the local concentration in the subplasmalemmal space may rise much earlier. At this time one may safely assume a role in PKG-mediated pp63/pf re-phosphorylation, with the effects outlined above.

11. Significance of multiple phosphorylation

We do not know whether pp63/pf molecules would be hierarchially phosphorylated. However, the emergence of several forms with different p*I*-values [27] suggests some heterogeneity in the overall phosphorylation. This may favour a hypothesis assuming an effect of the surface charge on structure-binding and availability in micro-spaces, e.g. in the subplasmalemmal space. Different phosphorylation forms may also possess different PGM activity. However, these aspects have not been analysed in any detail.

12. Potential role of pp63/pf/PGM de-phosphorylation during stimulated exocytosis

See Fig. 3A and B for a hypothetic scenario.

It is known from striated muscle cells that several glycolytic enzymes are enriched in the narrow space around terminal cisternae of the sarcoplasmic reticulum (SR), facing the cell membrane, i.e., the traverse tubules [66]. PGM is also enriched at these sites [67]. The PGM molecule has been assumed to govern the open/closed state of RyR-type Ca^{2+}-release channels depending on its phosphorylation, i.e., its dephospho-form was assumed to increase the probability of the open-state [66,67]. A similar claim has been raised for several intermediates of the glycolytic pathway [68].

May the activity of pp63/pf/PGM, therefore, be due to a direct, or rather to an indirect effect? Remarkably, RyR activity also depends on ATP [69] whose synthesis is compartmentalised in the triads of striated muscle cells [70]. Since RyRs can be activated (in secretory cells functioning with RyRs) by NAD-derived second messengers, the PGM activity of pp63/pf may exert an indirect function precisely along these lines. As mentioned, such messengers are cADPR [52,53,65] and $NAAD^+$ [55]. So far, for *Paramecium* this is hypothetic, because the second messengers activating RyR-like Ca^{2+}-release channels in these cells have to be determined as yet. Nevertheless, all our arguments suggest that cortical store activation occurs by some variants of RyR-type Ca^{2+}-release channels [5,20]. Accordingly, alveolar sacs react to the RyR activators, caffeine and 4-chloro-meta-cresol [5,20]. Ca^{2+} release from alveolar sacs precedes the superimposed Ca^{2+}-influx [17,20]. Experiments with a double mutant lacking any stimulated Ca^{2+}-influx support this concept [21].

From work with striated muscle, the assumption of direct regulation of RyR-type Ca^{2+}-channels by binding of glycolytic enzyme proteins [68,71] and/or PGM [67] has been derived. This would clearly be an effect in addition to any indirect effect via NAD-derived second messengers and from the established effect of ATP binding on RyR-type Ca^{2+}-channel activation [69]. Furthermore, ATP is also required to re-establish $[Ca^{2+}]_i$ homeostasis, e.g., by pumps, after a massive signal had been generated during exocytosis stimulation.

Another aspect concerns the SOC mechanism that participates in Ca^{2+}-signalling in *Paramecium* cells (see above). This can involve, in different cells, activation of Ca^{2+}-release channels not only of the inositol 1,4,5-trisphosphate receptor-type, but also others of the RyR-type [72,73], and a variety of TRP-like Ca^{2+}-influx channels with widely different activators [74]. Remarkably, it is known that some of these channels can be activated by cADPR [75] and that they are sensitive to the redox state [76].

What may be the relevance of all these aspects? Though many of these details in *Paramecium* still have to be explored on a molecular level, a potential scenario can be derived from the observations we made during synchronous exocytosis (Fig. 3A and B). To recall: during exocytosis stimulation, Ca^{2+}-signalling is roughly similar in exo^+ and exo^- *Paramecium* strains [23], as is ATP consumption [77]. However, when only exo^- cells are considerably compromised by a very slow ATP regeneration [37], is then an enzymatic role of pp63/pf, such as the PGM activity, plausible? Furthermore, since Ca^{2+}-mobilising second messengers formed from NAD may achieve only very low concentrations [54], could this be influenced by PGM activation and increased NAD^+/NADH formation? What might be its relevance for Ca^{2+}-signal generation and for re-establishment of $[Ca^{2+}]_i$ homeosasis, respectively? Our conclusions are as follows.

One has to consider that the volume where such messengers would have to be formed is very small; the subplasmalemmal space accounts only for 0.2% of the cell volume [5,29]. On the other hand, [ATP] decreases during exocytosis stimulation significantly by up to 27%, depending on the strain [77]. This occurs within 5 s, before the Ca^{2+}-pump in the cell membrane [78] and in the cortical stores [79–81] may be able to consume any substantial amount of ATP. Table 1 reveals the compromising effect on ATP regeneration in exo^- strains, in parallel to the inability to de-phosphorylate pp63/pf [37]. Mislocation of some key enzymes of energy metabolism can cause malfunction [51]. This could potentially explain both, the lack of pp63/pf dephosphorylation in exo^- *Paramecium* stains and their very sluggish ATP replenisment after AED stimulation [77]. ATP decay observed may by far exceed that occurring under "normal", physiological conditions. In fact, under such conditions, when a *Paramecium* cell wards off a predator by local trichocyst exocytosis, it releases only locally a limited extent of trichocysts—with a considerable effect on survival [82]. The ATP decay seen during maximal stimulation may, there-

fore, be the exaggeration of a smaller physiological effect. However, it may be precisely this effect which, on the one hand, allows us to trace regulation circles that otherwise would not be uncovered and, on the other hand, the *Paramecium* cell to survive.

As summarised in Fig. 3, the de-phosphorylation of pp63/pf occurring during stimulated exocytosis and its activity as PGM can be well reconciled with one or several aspects of signal transduction during synchronous exocytosis. This may encompass different aspects. For instance, in yeast cells, disruption of the PGM gene entails significant disturbance of the overall [Ca^{2+}] homeostasis [83]. pp63/pf/PGM may, thus, participate in Ca^{2+} regulation on a broad scale in widely different ways.

13. Conclusions

In this review, we try to connect data from molecular biology, cell biology, and biochemistry (Fig. 3A) to a hypothetic scenario (Fig. 3B). It may serve as a platform for further analysis of any more details in a fascinatingly complex network of overlaping functional cycles pertinent to Ca^{2+} regulation and regulation of Ca^{2+}-regulated processes. In essence we see the possible involvement of several overlaping signalling and metabolic steps. The involvement of widely spread key-players on the one hand, and some functional evidence on the other hand suggest a broad distribution of such phenomena among eukaryotic cells. In this regard, the *Paramecium* cell may prove itself again to be a suitable model system in particular due to its unsurpassed synchrony of dense-core vesicle exocytosis.

Acknowledgements

We gratefully acknowledge financial support by grants from the Deutsche Forschungsgemeinschaft to H.P., including grant PL 78/17 and Transregio-Sonderforschungsbereich, TR-SFB11, project C4.

References

[1] A. Verkhratsky, E.C. Toescu, Integrative Aspects of Calcium Signalling, Plenum Pr., London, 1998.
[2] M.J. Berridge, M.D. Bootman, H.L. Roderick, Calcium signalling: dynamics, homeostasis and remodelling, Nat. Rev. Mol. Cell Biol. 4 (2003) 517–529.
[3] R.D. Burgoyne, A. Morgan, Calcium sensors in regulated exocytosis, Cell Calcium 24 (1998) 367–376.
[4] R. Jahn, T. Lang, T.C. Südhof, Membrane fusion, Cell 112 (2003) 519–533.
[5] H. Plattner, N. Klauke, Calcium in ciliated protozoa: sources, regulation, and calcium regulated cell functions, Int. Rev. Cytol. 201 (2001) 115–208.
[6] H. Plattner, My favorite cell—*Paramecium*, BioEssays 24 (2002) 649–658.
[7] P. Dessen, M. Zagulski, R. Gromadka, H. Plattner, R. Kissmehl, E. Meyer, M. Bétermier, J.E. Schultz, J. Linder, R.E. Pearlman, C. Kung, J. Forney, B.H. Satir, J.L. Van Houten, A.-M. Keller, M. Froissard, L. Sperling, J. Cohen, *Paramecium* genome survey: a pilot project, Trends Genet. 17 (2001) 306–308.
[8] L. Sperling, P. Dessen, M. Zagulski, R.E. Pearlman, A. Migdalski, R. Gromadka, M. Froissard, A.-M. Keller, J. Cohen, Random sequencing of *Paramecium* somatic DNA, Eukaryot. Cell 1 (2002) 341–352.
[9] M. Zagulski, J.K. Nowak, A. LeMouel, M. Nowacki, A. Migdalski, R. Gromadka, B. Noel, I. Blanc, P. Dessen, P. Wincker, A.-M. Keller, J. Cohen, E. Meyer, L. Sperling, High coding density on the largest *Paramecium tetraurelia* somatic chromosome, Curr. Biol. 14 (2004) 1397–1404.
[10] H. Plattner, R. Kissmehl, Molecular apsects of membrane trafficking in *Paramecium*, Int. Rev. Cytol. 232 (2003) 185–216.
[11] H. Plattner, G. Knoll, R. Pape, Synchronization of different steps of the secretory cycle in *Paramecium tetraurelia*: trichocyst exocytosis, exocytosis-coupled endocytosis, and intracellular transport, in: H. Plattner (Ed.), Membrane Traffic in Protozoa, JAI Press, Greenwich (CT), London, 1993, pp. 123–148.
[12] G. Knoll, C. Braun, H. Plattner, Quenched flow analysis of exocytosis in *Paramecium* cells: time course, changes in membrane structure, and calcium requirements revealed after rapid mixing and rapid freezing of intact cells, J. Cell Biol. 113 (1991) 1295–1304.
[13] H. Kasai, Comparative biology of Ca^{2+}-dependent exocytosis: implications of kinetic diversity for secretory function, Trends Neurosci. 22 (1999) 88–93.
[14] H. Plattner, R. Kissmehl, Dense-core secretory vesicle docking and exocytotic membrane fusion in *Paramecium* cells, Biochim. Biophys. Acta 1641 (2003) 183–193.
[15] N. Stelly, J.P. Mauger, G. Keryer, M. Claret, A. Adoutte, Cortical alveoli of *Paramecium*: a vast submembraneous calcium storage compartment, J. Cell Biol. 113 (1991) 103–112.
[16] S. Länge, N. Klauke, H. Plattner, Subplasmalemmal Ca^{2+} stores of probable relevance for exocytosis in *Paramecium*. Alveolar sacs share some but not all characteristics with sarcoplasmic reticulum, Cell Calcium 17 (1995) 335–344.
[17] M. Hardt, H. Plattner, Sub-second quenched-flow/X-ray microanalysis shows rapid Ca^{2+} mobilization from cortical stores paralleled by Ca^{2+} influx during synchronous exocytosis in *Paramecium* cells, Eur. J. Cell Biol. 79 (2000) 642–652.
[18] J.L. Lovett, L.D. Sibley, Intracellular calcium stores in *Toxoplasma gondii* govern invasion of host cells, J. Cell Sci. 116 (2003) 3009–3016.
[19] C. Erxleben, H. Plattner, Ca^{2+} release from subplasmalemmal stores as a primary event during exocytosis in *Paramecium* cells, J. Cell Biol. 127 (1994) 935–945.
[20] N. Klauke, M.-P. Blanchard, H. Plattner, Polyamine triggering of exocytosis in *Paramecium* involves an extracellular Ca^{2+}/(polyvalent cation)-sensing receptor, subplasmalemmal Ca-store mobilization and store-operated Ca^{2+}-influx via unspecific cation channels, J. Membr. Biol. 174 (2000) 141–156.
[21] I. Mohamed, N. Klauke, J. Hentschel, J. Cohen, H. Plattner, Functional and fluorochrome analysis of an exocytotic mutant yields evidence of store-operated Ca^{2+} influx in *Paramecium*, J. Membr. Biol. 187 (2002) 1–14.
[22] H. Plattner, C. Braun, J. Hentschel, Facilitation of membrane fusion during exocytosis and exocytosis-coupled endocytosis and acceleration of "ghost" detachment in *Paramecium* by extracellular calcium. A quenched-flow/freeze-fracture analysis, J. Membr. Biol. 158 (1997) 197–208.
[23] N. Klauke, H. Plattner, Imaging of Ca^{2+} transients induced in *Paramecium* cells by a polyamine secretagogue, J. Cell Sci. 110 (1997) 975–983.

[24] D.M. Gilligan, B.H. Satir, Protein phosphorylation/dephosphorylation and stimulus-secretion coupling in wild type and mutant *Paramecium*, J. Biol. Chem. 257 (1982) 13903–13906.

[25] T.J. Murtaugh, D.M. Gilligan, B.H. Satir, Purification of and production of an antibody against a 63,000 M_r stimulus-sensitive phosphoprotein in *Paramecium*, J. Biol. Chem. 262 (1987) 15734–15739.

[26] E. Zieseniss, H. Plattner, Synchronous exocytosis in *Paramecium* cells involves very rapid ($\leq$1 s), reversible dephosphorylation of a 65-kD phosphoprotein in exocytosis-competent strains, J. Cell Biol. 101 (1985) 2028–2035.

[27] B. Höhne-Zell, G. Knoll, U. Riedel-Gras, W. Hofer, H. Plattner, A cortical phosphoprotein ('PP63') sensitive to exocytosis triggering in *Paramecium* cells. Immunolocalization and quenched-flow correlation of time course of dephosphorylation with membrane fusion, Biochem. J. 286 (1992) 843–849.

[28] T. Treptau, R. Kissmehl, J.D. Wissmann, H. Plattner, A 63 kDa phosphoprotein undergoing rapid dephosphorylation during exocytosis in *Paramecium* cells shares biochemical characteristics with phosphoglucomutase, Biochem. J. 309 (1995) 557–567.

[29] C. Erxleben, N. Klauke, M. Flötenmeyer, M.-P. Blanchard, C. Braun, H. Plattner, Microdomain Ca^{2+} activation during exocytosis in *Paramecium* cells. Superposition of local subplasmalemmal calcium store activation by local Ca^{2+} influx, J. Cell Biol. 136 (1997) 597–607.

[30] B.H. Satir, T. Hamasaki, R. Reichman, T.J. Murtaugh, Species distribution of a phosphoprotein (parafusin) involved in exocytosis, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 930–932.

[31] A.P. Andersen, E. Wyroba, M. Reichman, H. Zhao, B.H. Satir, The activity of parafusin is distinct from that of phosphoglucomutase in the unicellular eukaryote *Paramecium*, Biochem. Biophys. Res. Commun. 200 (1994) 1353–1358.

[32] S.V. Subramanian, E. Wyroba, A.P. Andersen, B.H. Satir, Cloning and sequencing of parafusin, a calcium-dependent exocytosis-related phosphoglycoprotein, Proc. Natl. Acad. Sci. U.S.A. 91 (1994) 9832–9836.

[33] N.D. Chilcoat, A.P. Turkewitz, In vivo analysis of the major exocytosis-sensitive phosphoprotein in *Tetrahymena*, J. Cell Biol. 139 (1997) 1197–1207.

[34] S.H. Matthiesen, S.M. Shenoy, K. Kim, R.H. Singer, B.H. Satir, Role of the parafusin orthologue, PRP1, in microneme exocytosis and cell invasion in *Toxoplasma gondii*, Cell. Microbiol. 5 (2003) 613–624.

[35] K. Hauser, R. Kissmehl, J. Linder, J.E. Schultz, F. Lottspeich, H. Plattner, Identification of isoforms of the exocytosis-sensitive phosphoprotein PP63/parafusin in *Paramecium tetraurelia* and demonstration of phosphoglucomutase activity, Biochem. J. 323 (1997) 289–296.

[36] R. Kissmehl, K. Hauser, M. Gössringer, M. Momayezi, N. Klauke, H. Plattner, Immunolocalization of the exocytosis-sensitive phosphoprotein, PP63/parafusin, in *Paramecium* cells using antibodies against recombinant protein, Histochem. Cell Biol. 110 (1998) 1–8.

[37] S. Müller, K. Diederichs, J. Breed, R. Kissmehl, K. Hauser, H. Plattner, W. Welte, Crystal structure analysis of the exocytosis-sensitive phosphoprotein, pp63/parafusin (phosphoglucomutase), from *Paramecium* reveals significant conformational variability, J. Mol. Biol. 315 (2001) 141–153.

[38] M. Kussmann, K. Hauser, R. Kissmehl, J. Breed, H. Plattner, P. Roepstorff, Comparison of in vivo and in vitro phosphorylation of the exocytosis-sensitive protein PP63/parafusin by differential MALDI mass spectrometric peptide mapping, Biochemistry 38 (1999) 7780–7790.

[39] C.B. Klee, P. Cohen, The calmodulin-regulated protein phosphatase, Mol. Aspects Cell Regul. 5 (1988) 225–248.

[40] F. Rusnak, P. Mertz, Calcineurin: form and function, Physiol. Rev. 80 (2000) 1483–1521.

[41] M. Momayezi, H. Kersken, U. Gras, J. Vilmart-Seuwen, H. Plattner, Calmodulin in *Paramecium tetraurelia*: localization from the in vivo to the ultrastructural level, J. Histochem. Cytochem. 34 (1986) 1621–1638.

[42] M. Momayezi, R. Kissmehl, H. Plattner, Quantitative immunogold localization of protein phosphatase 2B (calcineurin) in *Paramecium* cells, J. Histochem. Cytochem. 48 (2000) 1269–1281.

[43] M. Momayezi, C.J. Lumpert, H. Kersken, U. Gras, H. Plattner, M.H. Krinks, C.B. Klee, Exocytosis induction in *Paramecium tetraurelia* cells by exogenous phosphoprotein phosphatase in vivo and in vitro. Possible involvement of calcineurin in exocytotic membrane fusion, J. Cell Biol. 105 (1987) 181–189.

[44] R. Kissmehl, T. Treptau, H.W. Hofer, H. Plattner, Protein phosphatase and kinase activities possibly involved in exocytosis regulation in *Paramecium tetraurelia*, Biochem. J. 317 (1996) 65–76.

[45] R. Kissmehl, T. Treptau, B. Kottwitz, H. Plattner, Occurrence of a para-nitrophenyl phosphate-phosphatase with calcineurin-like characteristics in *Paramecium tetraurelia*, Arch. Biochem. Biophys. 344 (1997) 260–270.

[46] R.D. Hinrichsen, D. Fraga, C. Russell, The regulation of calcium in *Paramecium*, Adv. Sec. Mess. Phosphoprot. Res. 30 (1995) 311–338.

[47] A. Bandyopadhyay, D.W. Shin, J.O. Ahn, D.H. Kim, Calcineurin regulates ryanodine receptor/Ca^{2+}-release channels in rat, Biochem. J. 352 (2000) 61–70.

[48] D. Murray, A. Arbuzova, B. Honig, S. McLaughlin, The role of electrostatic and nonpolar interactions in the association of peripheral proteins with membranes, Curr. Top. Membr. 52 (2002) 271–302.

[49] G.N. Pierce, D.K. Philipson, Binding of glycolytic enzymes to cardiac sarcolemmal and sarcoplasmic reticular membranes, J. Biol. Chem. 260 (1985) 6862–6870.

[50] S.P.J. Brooks, K.B. Storey, Where is the glycolytic complex? A critical evaluation of present data from muscle tissue, FEBS Lett. 278 (1991) 135–138.

[51] P.A. Srere, H.R. Knull, Location–location–location, Trends Biochem. Sci. 23 (1998) 319–320.

[52] A. Galione, Cyclic ADP-ribose: a new way to control calcium, Science 259 (1993) 325–326.

[53] H.C. Lee, Cyclic ADP-ribose: a new member of a super family of signalling cyclic nucleotides, Cell. Sign. 6 (1994) 591–600.

[54] H.C. Lee, R. Aarhus, A derivative of NADP mobilizes calcium stores insensitive to inositol trisphosphate and cyclic ADP-ribose, J. Biol. Chem. 270 (1995) 2152–2157.

[55] H.C. Lee, A unified mechanism of enzymatic synthesis of two calcium messengers: cyclic ADP-ribose and NAADP, Biol. Chem. 380 (1999) 785–793.

[56] H. Plattner, M. Flötenmeyer, R. Kissmehl, N. Pavlovic, K. Hauser, M. Momayezi, N. Braun, J. Tack, L. Bachmann, Microdomain arrangement of the SERCA-type Ca^{2+}-pump (Ca^{2+}-ATPase) in subplasmalemmal calcium stores of *Paramecium* cells, J. Histochem. Cytochem. 47 (1999) 841–853.

[57] D. Vetter, R. Kissmehl, T. Treptau, K. Hauser, J. Kellermann, H. Plattner, Molecular identification of a calcium-inhibited catalytic subunit of casein kinase type 2 from *Paramecium tetraurelia*, Eukaryot. Cell 2 (2003) 1220–1233.

[58] R. Kissmehl, T.P. Krüger, T. Treptau, M. Froissard, H. Plattner, Identification of a multigene family encoding cGMP-dependent protein kinases in *Paramecium tetraurelia* cells, submitted for publication.

[59] M.L. Baker, I.I. Serysheva, S. Sencer, Y. Wu, S.J. Ludtke, W. Jiang, S.L. Hamilton, W. Chiu, The skeletal muscle Ca^{2+} release channel has an oxidoreductase-like domain, Proc. Natl. Acad. Sci. U.S.A. 99 (2002) 12155–12160.

[60] R. Kissmehl, T. Treptau, K. Hauser, H. Plattner, A novel, calcium-inhibitable casein kinase in *Paramecium* cells, FEBS Lett. 402 (1997) 227–235.

[61] G. Knoll, D. Kerboeuf, H. Plattner, A rapid calcium influx during exocytosis in *Paramecium* cells is followed by a rise in cyclic GMP within 1 s, FEBS Lett. 304 (1992) 265–268.

[62] J.E. Schultz, S. Klumpp, Cyclic nucleotides and calcium signaling in *Paramecium*, Adv. Cycl. Nucl. Phosphoprot. Res. 27 (1993) 25–46.

[63] J.U. Linder, P. Engel, A. Reimer, T. Krüger, H. Plattner, A. Schultz, J.E. Schultz, Guanylyl cyclases with the topology of mammalian adenylyl cyclases and an N-terminal P-type ATPase-like domain in *Paramecium, Tetrahymena* and *Plasmodium*, EMBO J. 18 (1999) 4222–4232.

[64] K.S. Murthy, G.M. Makhlouf, cGMP-mediated Ca^{2+} release from IP_3-insensitive Ca^{2+} stores in smooth muscle, Am. J. Physiol. 274 (Cell Physiol. 43) (1998) C1199–C1205.

[65] A. Galione, Cyclic ADP-ribose, the ADP-ribosyl cyclase pathway and calcium signalling, Mol. Cell. Endocrinol. 98 (1994) 125–131.

[66] R. Coronado, J. Morrissette, M. Sukhareva, D.M. Vaughan, Structure and function of ryanodine receptors, Am. J. Physiol. 266 (Cell Physiol. 35) (1994) C1485–C1504.

[67] Y.S. Lee, A.R. Marks, N. Gureckas, R. Lacro, B. Nadal-Ginard, D.H. Kim, Purification, characterization, and molecular cloning of a 60-kDa phosphoprotein in rabbit skeletal sarcoplasmic reticulum which is an isoform of phosphoglucomutase, J. Biol. Chem. 267 (1992) 21080–21088.

[68] H. Kermode, W.M. Chan, A.J. Williams, R. Sitsapesan, Glycolytic pathway intermediates activate cardiac ryanodine receptors, FEBS Lett. 431 (1998) 59–62.

[69] G. Meissner, Adenine nucleotide stimulation of Ca^{2+}-induced Ca^{2+}-release in sarcoplasmic reticulum, J. Biol. Chem. 259 (1984) 2365–2374.

[70] J.W. Han, R. Thieleczek, M. Varsány, L.M.G. Heilmeyer, Compartmentalized ATP synthesis in skeletal muscle triads, Biochemistry 31 (1992) 377–384.

[71] J. Hüser, Y.G. Wang, K.A. Sheehan, F. Cifuentes, S.L. Lipsius, L.A. Blatter, Functional coupling between glycolysis and excitation-contraction coupling underlies alternans in cat heart cells, J. Physiol. 524 (2000) 795–806.

[72] K. Venkatachalam, D.B. Van Rossum, R.L. Patterson, H.T. Ma, D.L. Gill, The cellular and molecular basis of store-operated calcium entry, Nat. Cell Biol. 4 (2002) E263–E272.

[73] J. Ma, Z. Pan, Retrograde activation of store-operated calcium channel, Cell Calcium 33 (2003) 375–384.

[74] R. Padinjat, S. Andrews, TRP channels at a glance, J. Cell Sci. 117 (2004) 5707–5709.

[75] M.D. Cahalan, Channels as enzymes, Nature 411 (2001) 542–543.

[76] Y. Hara, M. Wakamori, M. Ishii, E. Maeno, M. Nishida, T. Yoshida, H. Yamada, S. Shimizu, E. Mori, J. Kudoh, N. Shimizu, H. Kurose, Y. Okada, K. Imoto, Y. Mori, LTRPC2 Ca^{2+}-permeable channel activated by changes in redox status confers susceptibility to cell death, Mol. Cell 9 (2002) 163–173.

[77] J. Vilmart-Seuwen, H. Kersken, R. Stürzl, H. Plattner, ATP keeps exocytosis sites in a primed state but is not required for membrane fusion. An analysis with *Paramecium* cells in vivo and in vitro, J. Cell Biol. 103 (1986) 1279–1288.

[78] M.V. Wrigth, J.L. Van Houten, Characterization of a putative Ca^{2+}-transporting Ca^{2+}-ATPase in the pellicles of *Paramecium tetraurelia*, Biochim. Biophys. Acta 1029 (1990) 241–251.

[79] R. Kissmehl, S. Huber, B. Kottiwtz, K. Hauser, H. Plattner, Subplasmalemmal Ca-stores in *Paramecium tetraurelia*. Identification and characterisation of a sarco(endo)plasmic reticulum-like Ca^{2+}-ATPase by phosphoenzyme intermediate formation and its inhibition by caffeine, Cell Calcium 24 (1998) 193–203.

[80] K. Hauser, N. Pavlovic, R. Kissmehl, H. Plattner, Molecular characterization of a sarco(endo)plasmic reticulum Ca^{2+}-ATPase gene from *Paramecium tetraurelia* and localization of its gene product to sub-plasmalemmal calcium stores, Biochem. J. 334 (1998) 31–38.

[81] K. Hauser, N. Pavlovic, N. Klauke, D. Geissinger, H. Plattner, Green fluorescent protein-tagged sarco(endo)plasmic reticulum Ca^{2+}-ATPase overexpression in *Paramecium* cells: isoforms, subcellular localization, biogenesis of cortical calcium stores and functional aspects, Mol. Microbiol. 37 (2000) 773–787.

[82] G. Knoll, B. Haacke-Bell, H. Plattner, Local trichocyst exocytosis provides an efficient escape mechanism for *Paramecium* cells, Eur. J. Protistol. 27 (1991) 381–385.

[83] L. Fu, A. Miseta, D. Hunton, R.B. Marchase, D.M. Bedwell, Loss of the major isoform of phosphoglucomutase results in altered calcium homeostasis in *Saccharomyces cerevisiae*, J. Biol. Chem. 275 (2000) 5431–5440.