BIOCHEMICAL JOURNAL LETTERS

Rapsyn's knobs and holes: eight tetratrico peptide repeats

It has been reported recently that postsynaptic specialization fails to develop at neuromuscular junctions in rapsyn-deficient mice [1]. Mice unable to express rapsyn showed greatly reduced clustering of nicotinic acetylcholine receptors (AChRs) at these junctions, and synaptic concentrations of other components of the AChR-associated cytoskeleton, namely utrophin (the 400 kDa dystrophin homologue), α-syntrophin and dystroglycan, were shown to be greatly reduced [1]. This suggests that rapsyn is essential for the organization of the postsynaptic cytoskeleton and supports a proposal that rapsyn acts as a molecular link between AChRs and the dystrophin–dystroglycan complex [2]. Rapsyn (**r**eceptor-**a**ssociated **p**rotein at the **syn**apse, also known as the '45k protein') was first identified in *Torpedo californica* postsynaptic membranes and is thought to contain a C-terminal C1-like zinc-finger motif and a leucine zipper; otherwise its sequence has not been shown to be similar to that of any other protein [3]. Here we demonstrate that rapsyn contains eight putative tetratrico peptide repeats (TPRs).

A BLAST [4] search of databases with the murine rapsyn sequence yielded apparently significant (P values ranging between 2×10^{-12} and 1×10^{-2}) similarities between rapsyn and a variety of TPR-containing proteins, including yeast 70 kDa mitochondrial outer-membrane protein [5], *Synechocystis* sp. and *Caenorhabditis elegans* hypothetical proteins, human and mouse extendins (IEF SSP 3521) [6] and the *Vibrio anguillarum virC* gene product (VirC) (Figure 1). TPRs are degenerate 34-amino acid repeated motifs that are widespread in evolution and are present in proteins involved in a diverse set of cellular processes [8,9]. They have a high probability of forming amphipathic helices containing seven consensus residues that are postulated to be involved in helix–helix packing by a 'knob and hole' mechanism [8]. The rapsyn putative TPR sequences are consistent with this pattern of conservation (Figure 1). The TPR sequences in rapsyn, VirC and *Synechocystis* sp. and *C. elegans* hypothetical proteins appear to be variations of the more recognized TPR theme [9] in that they lack helix-breaking prolines at position 32, and contain linker sequences. As such they are more similar to the TPRs in soluble *N*-ethylmaleimide-sensitive fusion protein ('NSF') attachment proteins ('SNAPs') [10]. The putative TPR3 sequence, although coincident with a proposed

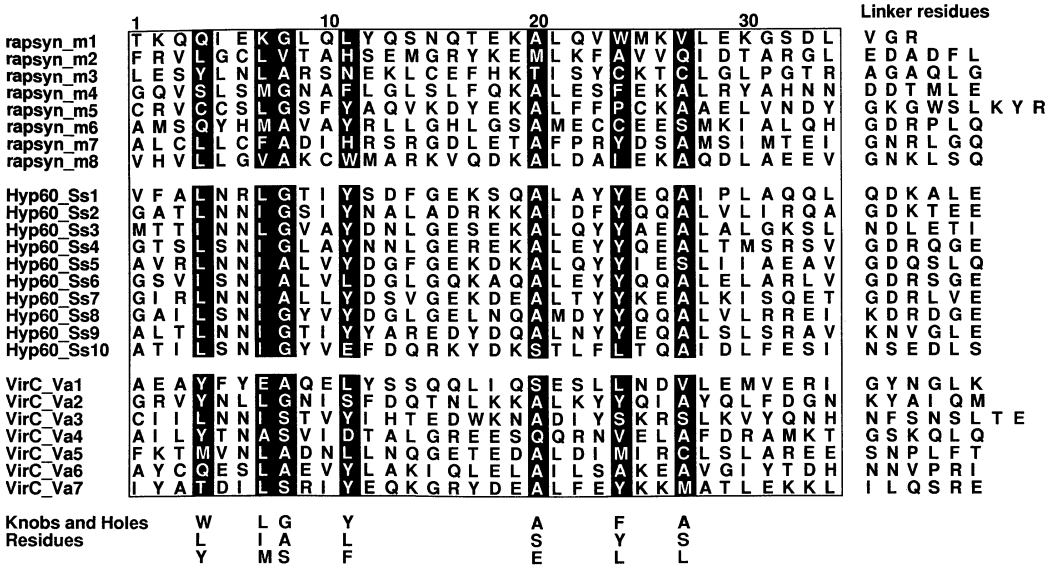

Figure 1 Multiple alignment of putative TPR sequences from murine rapsyn, the *Synechocystis* sp. hypothetical protein (Hyp60) and *V. anguillarum* VirC (accession codes SwissProt: [sw:] Ps43_Mouse, Genbank: [gb:] Sycsllhh_60 and gb:Vau17054_2 respectively)

Linker sequences are shown following the alignment. Rapsyns from human, *Xenopus laevis* and *T. californica* are highly similar to the murine sequence (amino acid identities > 70%) and may be aligned without the requirement of insertions or deletions. Positions where amino acids form the hole (positions 4, 7, 8 and 11) and the knob (positions 20, 24 and 27) of the TPR are shown as white-on-black; the three most frequent residues at these positions in established TPRs [9] are shown beneath. A search [4] of current databases for murine rapsyn homologues yielded sequence similarities with regions of proteins shown elsewhere to contain TPRs [these included yeast 70 kDa mitochondrial outer-membrane protein, sw:OM70_YEAST (seven TPRs), calculated probability of four alignment blocks arising by chance: $P(n = 4) = 6.8 \times 10^{-4}$; and human extendin (five TPRs), sw:IEFS_HUMAN, $P(n = 4) = 7.9 \times 10^{-4}$] and others that were shown here to possess TPRs [Hyp60 (ten TPRs), $P(n = 2) = 1.8 \times 10^{-12}$; *C. elegans* hypothetical protein (eight TPRs), gb:Celf32a6_2, $P(n = 3) = 2.7 \times 10^{-9}$; and VirC (seven TPRs), $P(n = 2) = 1.3 \times 10^{-2}$]. Three-way alignments of each putative rapsyn TPR with one Hyp60 TPR and one VirC TPR yielded calculated [15] P values of between 10^{-3} and 10^{-7}, demonstrating the significance of their similarities.

leucine zipper motif [3], is not necessarily inconsistent with it, since leucine zipper motifs and TPRs share a common secondary-structural element, namely the α-helix.

What functions might these eight rapsyn TPRs serve? It is known that TPRs bind both to each other and to non-TPR-containing proteins [11], and their presence in rapsyn suggests that they may form a scaffold upon which the components of the postsynaptic cytoskeleton assemble. A potential AChR-binding function for these TPRs is suggested by a rapsyn variant containing all eight putative TPRs and a disrupted zinc-finger motif, which retains affinity for AChR yet is less able to form AChR clusters [12].

Gautam et al. [1] discussed additional consequences of rapsyn elimination in mice: nerve endings formed long branches lacking the distinct arbours typical of normal terminals and numerous axons ran between muscle fibres without forming specialized contacts. One suggested explanation for these abnormalities was the loss of a retrograde signalling mechanism from the post-synaptic apparatus [1,3]. A recent report demonstrated that neuronal nitric oxide synthase (nNOS) localizes to the post-synaptic cytoskeleton by binding dystrophin and possibly utrophin [13]. NO is known to act as a retrograde messenger at neuromuscular junctions [14], and thus loss of targeted nNOS activity at the postsynaptic junction is a possible cause for the observed aberrant presynaptic differentiation. If this prediction proves to be correct, then the putative rapsyn TPRs may mediate clustering of AChRs and the assembly of nNOS–dystrophin/utrophin complexes at the postsynaptic junction, thereby targeting signalling mechanisms to their appropriate sites of action.

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1 Gautam, M., Noakes, P. G., Mudd, J., Nichol, M., Chu, G. C., Sanes, J. R. and Merlie, J. P. (1995) Nature (London) **377**, 232–236
2 Apel, E. D., Roberds, S. L., Campbell, K. P. and Merlie, J. P. (1995) Neuron **15**, 115–126
3 Froehner, S. C. and Fallon, J. R. (1995) Nature (London) **377**, 195–196
4 Altschul, S. F., Boguski, M. S., Gish, W. and Wootton, J. C. (1994) Nature (London) **6**, 119–129
5 Boguski, M. S., Sikorski, R. S., Hieter, P. and Goebl, M. (1990) Nature (London) **346**, 114
6 Honoré, B., Leffers, H., Madsen, P., Rasmussen, H. H., Vandekerckhove, J. and Celis, J. E. (1992) J. Biol. Chem. **267**, 8485–8491
7 Milton, D. L., Norqvist, A. and Wolf-Watz, H. (1995) Gene **164**, 95–100
8 Goebl, M. and Yanagida, M. (1991) Trends Biochem. Sci. **16**, 173–177
9 Sikorski, R. S., Boguski, M. S., Goebl, M. and Hieter, P. (1990) Cell **60**, 307–317
10 Ordway, R. W., Pallanck, L. and Ganetzky, B. (1994) Trends Biochem. Sci. **19**, 530–531
11 Lamb, J. R., Tugendreich, S. and Hieter, P. (1995) Trends Biochem. Sci. **20**, 257–259
12 Scotland, P. B., Colledge, M., Melnikova, I., Dai, Z. and Froehner, S. C. (1993) J. Cell Biol. **123**, 719–728
13 Brenman, J. E., Chao, D. S., Xia, H., Aldape, K. and Bredt, D. S. (1995) Cell **82**, 743–752
14 Wang, T., Xie, Z. and Lu, B. (1995) Nature (London) **374**, 262–266
15 Schuler, G. D., Altschul, S. F. and Lipman, D. J. (1991) Proteins Struct. Funct. Genet. **9**, 180–190

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Capacitative Ca^{2+} influx and a diffusible influx factor

The recent excellent review by Berridge [1] well summarized the current lack of consensus about the mechanism of capacitative Ca^{2+} entry. A central unresolved problem is whether a diffusible Ca^{2+} influx factor exists or is required for capacitative influx. Recently, Clapham [2] gave a pessimistic view of the status of the factor isolated by Randriamampita and Tsien [3], as being a physiological Ca^{2+}-influx factor. He instead highlighted the possibility of mechanisms in which diffusion of a store–plasma membrane messenger were not required. In the review by Berridge [1] this aspect was also highlighted, with the main Figure showing a 'gap' between the Ca^{2+} store (presumably endoplasmic reticulum) and plasma membrane as just 20 nm. The gap was small enough to be spanned by a single InsP_3 receptor. One could argue that such a close conjunction of the two membranes negates the need, not only for a diffusible Ca^{2+}-influx factor, but also for diffusible InsP_3. However, a gap of such small scale may not always exist.

In neutrophils, transmembrane Ca^{2+} influx is increased by any of a number of manoeuvres, whether experimental (thapsigargin, ionomycin etc.) or physiological (fMet-Leu-Phe), which release Ca^{2+} from stores [4,5]. This cell type thus demonstrates the classic capacitative Ca^{2+} influx seen in a number of other cell types. The neutrophil, however, provides an unusual opportunity to visualize the 'lay-out' of the components responsible for this 'Ca^{2+} store–plasma membrane communication', as unlike many other cell types, they have few intracellular membranes and little endoplasmic reticulum (Figure 1). The site of the stored Ca^{2+} which is released by formylated peptide has been identified as juxta-nuclear [6]. The 'vestigial' endoplasmic reticulum which remains in these terminally differentiated cells, also nestles close to the nucleus, and is thus the most obvious organelle candidate for a Ca^{2+} store (Figure 1). It is difficult to identify or envisage any structural route from this organelle deep in the heart of the cell to the plasma membrane. Diffusion of a factor from this site remains the most likely route for signalling the plasma membrane.

Furthermore, we have recently shown that, with fMet-Leu-Phe as stimulus, there is a measurable minimum delay of 75 ms

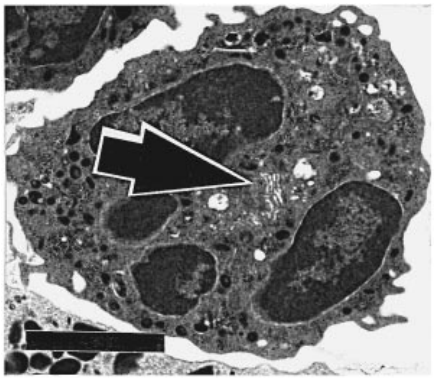

Figure 1 Location of the 'endoplasmic reticulum' in neutrophils

The electron micrograph shows a section through a human neutrophil. The main features are the prominent nuclear lobes and the membranous, smooth-endoplasmic-reticulum-like structure at the centre of the cell (marked by an arrow). The position of the latter correlates with a site of Ca^{2+} release. No other 'endoplasmic-reticulum' membranes are observable. The scale bar represents 4 μm.

before global cytosolic free Ca^{2+} concentration rises [7]. Such a delay is consistent with diffusion of $Ins P_3$ from the plasma membrane and a Ca^{2+}-influx factor from such a centrally located Ca^{2+} store [7,8]. With such a 'lay-out' Ca^{2+} signalling by this route cannot produce localized Ca^{2+} signalling within the cell. It would be difficult to imagine how random diffusion of $Ins P_3$ to the store and a Ca^{2+}-influx factor from this centrally located store would produce localized, rather than global, Ca^{2+} changes. In contrast with formylated peptide receptor occupancy, however, cross-linking of receptors for Fc or for integrin *does* trigger localized Ca^{2+} signals and cell responses [9–11] in neutrophils. These localized Ca^{2+} signals result from liberation of Ca^{2+} from different storage sites near the plasma membrane. The release of Ca^{2+} from these sites, which have been visualized by confocal and conventional Ca^{2+} imaging [9–12], is not accompanied by the near-simultaneous influx of extracellular Ca^{2+} [7–12], and may therefore not involve capacitative Ca^{2+} influx. These Ca^{2+} storage sites may be those identified as containing calreticulin [13] and which are visualized in neutrophils to coalesce around the newly formed phagosome [14].

Part of the problem in reaching a consensus about the mechanism of capacitative Ca^{2+} influx may thus arise from the differences which exist between different cell types, and between different signalling systems which co-exist within a particular cell type. The prospects for the future of the diffusible Ca^{2+}-influx factor theory, therefore, may not be as bleak as has recently been made out.

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1 Berridge, M. J. (1995) Biochem. J. **312**, 1–11
2 Clapham, D. E. (1995) Nature (London) **375**, 634–635
3 Randriamampita, C. and Tsien, R. Y. (1993) Nature (London) **364**, 809–814
4 Montero, M., Alvarez, J. and Garcia-Sancho, J. (1991) Biochem. J. **277**, 73–79
5 Scharff, O. and Foder, B. (1993) Physiol. Rev. **73**, 547–582
6 Davies, E. V., Hallett, M. B. and Campbell, A. K. (1991) Immunology **73**, 228–234
7 Pettit, E. J. and Hallett, M. B. (1995) Biochem. J. **310**, 445–448
8 Hallett, M. B. and Pettit, E. J. (1996) J. Theor. Biol., in the press
9 Davies, E. V., Campbell, A. K. and Hallett, M. B. (1994) Immunology **82**, 57–62
10 Pettit, E. J. and Hallett, M. B. (1995) Eur. J. Clin. Invest. **25**, A28
11 Petersen, M., Williams, J. D. and Hallett, M. B. (1993) Immunology **80**, 157–159
12 Davis, W. D., Sage, S. O. and Allen, J. M. (1994) Cell Calcium **16**, 29–36
13 Volpe, P., Krause, K.-H., Hashimoto, S., Zorzato, F., Pozzan, T., Meldolsei, J. and Lew, D. P. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 1091–1095
14 Stendahl, O., Krause, K.-H., Krischer, J., Jerstrom, P., Theler, J.-M., Clark, R. A., Carpentier, J.-L. and Lew, D. P. (1994) Science **265**, 1439–1441

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Capacitative Ca^{2+} entry – sifting through the evidence for CIF

Hallett, Pettit and Davies [1] have correctly highlighted the importance of the topographical arrangement of Ca^{2+} stores relative to the plasma membrane as a key issue in the current debate concerning the mechanism of capacitative Ca^{2+} entry. The conformational coupling model which I outlined in detail in my recent review [2] requires a part of the Ca^{2+} store to come into close contact with the plasma membrane so that the $Ins P_3$ receptor can interact directly with the putative store-operated channel (SOC). The alternative proposal that coupling is achieved by a calcium influx factor (CIF) [3,4], a view favoured by Hallett and his colleagues [5,6], does not require such close juxtaposition. It has proved very difficult to obtain unequivocal evidence to distinguish between these two mechanisms, even though they are so different.

Hallett et al. [1] have used structural and temporal arguments to support the CIF hypothesis. For example, they describe how neutrophils can display capacitative Ca^{2+} entry in the apparent absence of an association between the Ca^{2+} stores and the plasma membrane [1]. This argument is based on the assumption that there is a single discrete $Ins P_3$-sensitive store, whereas it has been argued previously that cells may have separate stores, one close to the membrane responsible for controlling the entry of Ca^{2+}, which then charges up the deeper stores that generate Ca^{2+} spikes [7]. As the stores near the membrane are likely to be small, it would be difficult to observe the Ca^{2+} being released in imaging experiments, and they certainly would not be apparent in the low-magnification electron micrograph presented by Hallett et al. [1]. This micrograph also indicates a central localization of the endoplasmic reticulum which appears to contradict a previous report which suggested that the store was localized "…between the nuclear lobes and the plasma membrane" [8]. Also, Ca^{2+} imaging of activated neutrophils revealed a localized elevation of Ca^{2+} in close vicinity to the plasma membrane, indicating that stores may indeed come close to the cell surface [8,9]. Furthermore, this micrograph fails to reveal the separate Ca^{2+} stores which produce the localized Ca^{2+} signals in response to cross-linking Fc or integrin receptors [10]. It is difficult, therefore, to use such structural evidence to argue against the existence of the small subset of stores near the plasma membrane which have been implicated in conformational coupling [2].

In addition to these topographical aspects, Hallett et al. [1] have also introduced a temporal argument to support the existence of a diffusible factor. They argue that the CIF theory may account for the delay (minimum 75 ms; average 530 ms) between the arrival of fMet-Leu-Phe and the global activation of Ca^{2+} entry by CIF. Such a mechanism would require that the agonist generates $Ins P_3$; the latter must then diffuse to the internal store to stimulate Ca^{2+} release, and the empty store must produce CIF, which must then diffuse back to the membrane to activate entry all within a minimum of 75 ms. While not impossible, it is difficult to envisage how all these events comprising at least one enzymic step could be achieved in less than a second. It may be argued, equally convincingly, that such a short latency may rule out the CIF theory and support the notion that entry is controlled by events localized at the cell membrane. Another explanation for latency is that it results from a temporary inhibition of entry brought on by the localized elevation of Ca^{2+} as the stores near the membrane are emptied [2].

As this response to the letter by Hallett et l. [1] clearly indicates, our interpretation of data is often influenced by our subjective attachment to the various models of capacitative calcium entry. Even though I have interpreted the neutrophil data somewhat differently to Hallett et al. [2], I certainly concur with their conclusion that the CIF hypothesis is still viable. What is so interesting about this hypothesis is that there is a clearly defined prediction: empty Ca^{2+} stores produce a diffusible factor that controls Ca^{2+} entry. Before the hypothesis can be accepted, however, we need to know what this factor is and how it is generated by empty stores.

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1 Hallett, M. B., Pettit, E. J. and Davies, E. V. (1996) Biochem. J. **314**, 1054–1055
2 Berridge, M. J. (1995) Biochem. J. **321**, 1–11
3 Randriamampita, C. and Tsien, R. Y. (1993) Nature (London) **364**, 809–814

4 Thomas, D. and Hanley, M. R. (1995) J. Biol. Chem. **270**, 6429–6432
5 Davies, E. V., Campbell, A. K. and Hallett, M. B. (1994) Immunology **82**, 57–62
6 Davies, E. V. and Hallett, M. B. (1995) Biochem. Biophys. Res. Commun. **206**, 348–354
7 Berridge, M. J. (1992) Adv. Second Messenger Phosphoprotein Res. **26**, 211–223
8 Davies, E. V., Hallett, M. B. and Campbell, A. K. (1991) Immunology **73**, 228–234
9 Pettit, E. J. and Hallett, M. B. (1995) Biochem. J. **310**, 445–448
10 Petersen, M., Williams, J. D. and Hallett, M. B. (1993) Immunology **80**, 157–159

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