Concepts of cAMP signalling have changed dramatically from the linear cascades of just a few years ago, with the realization that numerous cellular processes affect this motif. These influences include other signalling pathways – most significantly Ca\(^{2+}\), scaffolding proteins (which are themselves variously regulated) to organize the elements of the pathway, and subcellular targeting of components. An obvious implication of this organization is that global measurements of cAMP may trivialize the complexity of the cAMP signals and obscure the regulation of targets. In this presentation, current developments on the targeting and assembly of ACs (adenylate cyclases) and their delivery to selected raft or non-raft domains of the plasma membrane will be discussed, along with the susceptibility of raft-targeted ACs to very discrete modes of increases in the intracellular Ca\(^{2+}\) concentration. Single-cell explorations of cAMP dynamics, as measured with cyclic nucleotide-gated channels, are also described in this paper, particularly as applied to cells in which the composition of AKAP (A-kinase anchoring protein)–PKA (protein kinase A)–PDE (phosphodiesterase) assemblies is probed by RNA interference ablation of defined AKAPs.

Assembly of ACs (adenylate cyclases)

Membrane-bound AC isoforms are large proteins of approx. 120–140 kDa that share a common secondary structure comprising an intracellular N-terminus, two tandem six-TM (transmembrane) cassettes separated by a cytoplasmic loop termed the C1 domain and a C-terminal cytoplasmic C2 domain (Figure 1) [1]. The ATP-binding C1a and C2a domains are the most conserved regions between AC isoforms and their interaction forms the catalytic core. The C1a and C2a domains can be expressed in vitro to form a G\(_\alpha\)S- and forskolin-stimulable enzyme [1]. However, in the intact cell, these cytosolic domains do not exhibit sufficiently high affinity for each other to form a stable catalytic core, and they are rapidly degraded in the absence of TMDs (TM domains). Precise intra- and inter-molecular associations between the TMDs are increasingly being recognized to play a role in regulating the formation of the catalytic core, membrane trafficking and dimer/oligomer assembly. The two TMDs have a high affinity for each other and this stabilizes a functional catalytic core [2,3]. Although there is little sequence identity in the TMDs, they must be internally complementary, since AC activity can only be generated by two TMD cassettes that are derived from the same AC isoform [3].

Intramolecular interactions between the two TMDs have been examined in some detail by Gu et al. [2], who applied FRET (fluorescence resonance energy transfer) analysis to fluorescently tagged AC8 constructs. This approach revealed the importance of intramolecular interactions in the targeting of AC8 to the plasma membrane in living cells. When either six-pass TMD was expressed alone, localization was entirely intracellular. However, upon co-transfection of the partner six-pass TMD, the couple dimerized and was targeted to the plasma membrane. This effect was quite independent of the cytoplasmic domains. FRET analysis confirmed that the two TMDs form a tight complex, which is essential for catalytic activity, since co-expression of the separate halves of AC8 (i.e. Nt-TMD1-C1 and TMD2-C2) resulted in a plasma membrane-localized, Ca\(^{2+}\)–stimulable AC8. Therefore precise intramolecular associations, especially...
Figure 1 | Assembly of AC

Interactions between the TMDs of AC (Tm1 and Tm2) compensate for the weak affinity of the catalytic domains (C1/C2). Adapted from Current Biology 11: Gu, C., Sorkin, A. and Cooper, D.M.F. ‘Persistent interactions between the two transmembrane clusters dictate the targeting and functional assembly of adenylyl cyclase.’, pp. 185–190. © 2001 with permission from Elsevier.

between the two transmembrane cassettes, are an important feature both in terms of targeting and allowing the catalytic C1a and C2a domains to interact [2].

The concept of AC dimerization predates the now widely accepted GPCR (G-protein-coupled receptor) dimerization. A whole array of distinct, but complementary, approaches points to the conclusion that AC function depends on dimerization (or oligomerization). Structurally, ACs are members of the ABC (ATP-binding cassette) superfamily, which includes the CFTR (cystic fibrosis transmembrane conductance regulator), P-glycoprotein and other ATP-binding transporters, all of which form multimeric assemblies [4]. By analogy, it would not be unexpected that ACs would exist as higher order multimeric structures. Indeed, early work by Rodbell and co-workers [5] using target size analysis suggested that a dimer is the minimal size of an activated AC. Further hydrodynamic analysis of detergent solubilized preparations of AC suggested that monomer/dimer transitions could occur in the catalytic unit [6]. In vitro, a C-terminally truncated active AC1 and an inactive full-length AC1 are co-immunoprecipitated by a C-terminus-specific AC1 antibody and the precipitate displays AC activity, suggesting that AC1 dimerizes [7]. Previous FRET studies with AC8 showed that co-expression of the second TMD tagged with either CFP (cyan fluorescent protein) or YFP (yellow fluorescent protein) resulted in the formation of a tight homodimer, as determined by FRET analysis. However, the interacting second TM cassette is not targeted to the plasma membrane, unless the first TM cassette is also co-expressed [8]. In support of the functional importance of AC8 dimerization, co-expression of an inactive AC8 mutant decreases the activity of co-expressed wild-type AC8, without affecting the expression level or targeting of wild-type AC8 [8]. This suggests that an active AC8 requires a dimer/oligomer structure, with dimerization mediated through interactions of the second TMD (Figure 1). Adopting an in vitro enzyme kinetic analysis, Chen-Goodspeed et al. [9] addressed the activation of AC5 by Goα. They found that AC5 displayed a clear co-operative activation by Goα, with a Hill coefficient of 1.4. They suggested that the most likely mechanism for Goα binding to AC5 was through a dimer, since the co-operative nature of binding was fitted best by a two-site model and it is unlikely that two Goα-binding sites occur on a single AC monomer. AC6, which is highly similar to AC5, has also been suggested to form homodimers, as N-terminally FLAG-tagged AC6 co-immunoprecipitates with N-terminally Myc-tagged AC6 and vice versa [10]. Like AC8, the TMDs rather than the cytoplasmic regions were found to be involved in homodimerization, but an important role for the first TM cassette was identified.

A number of studies have also suggested that AC heterodimerization can occur, but it remains experimentally quite challenging to assess the significance of such interactions [8,10].

Targeting of cyclases: protein–protein interactions in lipid rafts

It is becoming increasingly evident that many signalling molecules are restricted in their placement in the plasma membrane to so-called lipid raft domains. These include GPCRs, G-protein subunits and effectors, including ion channels and certain ACs [11]. Indeed it turns out to be an essential determinant of the sensitivity of Ca2+-sensitive ACs to CCE (capacitative Ca2+ entry) that they be localized in lipid rafts. If cellular cholesterol is depleted, these enzymes are no longer sensitive to CCE [12]. Although there is not a consensus on the significance of or role played by lipid rafts [13], rigorous efforts to establish the association or otherwise of some proteins strongly identifies some as permanent residents – and not others [14]. With regard to ACs, a recent exploration of the domains targeting AC5 and AC8 to rafts – unlike AC7, which is excluded from rafts – strongly implicated the cytosolic domains [15]. This was somewhat surprising, since intuitively lipid solubility might have been expected to be determined by the hydrophobic TM-spanning domains. These observations lead to the conclusion that perhaps it is by protein–protein association of the ACs with other raft-embedded proteins that they are retained in the rafts [15].

Regulation by CCE

A remarkable feature of Ca2+-sensitive ACs is their dependence on CCE in non-excitable cells. They are not regulated by release from intracellular stores – nor by the so-called
non-CCE in the same cells. They are also not regulated by ionophore-mediated Ca\(^{2+}\) entry [4]. However, these enzymes are also regulated by co-expressed CNG (cyclic nucleotide-gated) channels [16] (which, incidentally, and perhaps significantly, are also targeted to lipid rafts [17] and by OAG (1-oleoyl-2-acetyl-sn-glycerol)-stimulated Ca\(^{2+}\) entry (unpublished work)). In excitable cells, Ca\(^{2+}\)-sensitive ACs are regulated not only by the very robust entry of Ca\(^{2+}\) through voltage-gated Ca\(^{2+}\) channels, but remarkably also by the very modest degree of CCE that can be detected in such cells [18]. Finally, the close apposition of ACs with CCE channels is underscored by the efficacy of the fast Ca\(^{2+}\) chelator BAPTA/AM [bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid tetrakis(acetoxymethyl ester)] at muting the regulation, compared with the ineffectiveness of EGTA/AM (EGTA acetoxymethyl ester) in in vivo experiments [19]. The precise mechanism underlying this dependence remains a subject of investigation.

Microdomains of cAMP

The inaccessibility of ACs to Ca\(^{2+}\) entering via ionophores raises (at least) two possibilities. (i) Ca\(^{2+}\) is not entering the cell near the ACs. There are some data showing that ionophores are excluded from cholesterol-rich membranes and that cholesterol limits Ca\(^{2+}\) transport by ionophore in artificial bilayers [20–24]. (ii) The nature of the ionophore-mediated Ca\(^{2+}\) rise does not mimic the Ca\(^{2+}\) rise associated with opening and closing of ion channels and the large transitions in Ca\(^{2+}\) involved. The first option suggests a microdomain surrounding the cyclase, which is reminiscent of the microdomain proposed to surround CCE channels [25]. Very recent experiments from the laboratory underscore the possibility of a physical microdomain around the cyclase, in that it is known that ACs are insulated from global transitions in pH by the presence (in rafts) of the sodium/hydrogen exchanger, NHE1 (Na\(^+\)/H\(^+\) exchanger isoform 1), which protects the AC from acid transitions (unpublished work). The second option points to a more kinetic microdomain in terms of the pattern of Ca\(^{2+}\) transients near a channel, which are buffered or diffused at greater distances.

Single-cell cAMP

The most direct means of pursuing the existence of a microdomain of cAMP, which differs from that of cAMP in the broad cytosol, is to try to measure cAMP in the immediate environment of AC. We have developed CNG channels, mutated to exhibit high affinity for cAMP, to report cAMP dynamically at the plasma membrane [26,27]. It became immediately apparent that cAMP kinetics were quite transient at the plasma membrane, compared with a gradual accumulation in the cytosol. Extensive probing with pharmacological agents and siRNAs (small interfering RNAs) directed against selective AKAPs (A-kinase anchoring proteins) has now led us to conclude that the vicinity of AC in HEK-293 cells (human embryonic kidney 293 cells) includes the AKAP, gravin and PKA (protein kinase A), which rapidly activate PDE4 (phosphodiesterase 4) to produce the transient cAMP response (Figure 2). cAMP that escapes the PDE slowly accumulates in the cytosol [28].

Summary

More than 20 years ago, Buxton and Brunton [29] made a series of observations in cardiomyocytes that could only be explained by separate pools of cAMP in the same cell. These observations have stood the test of time and have since been validated by others – most notably by Jurevics and Fischmeister [30]. However, ‘compartmentalization’ has sometimes been invoked to explain puzzling findings with little concrete evidence, with the result that the concept of microdomains became somewhat suspect. Of course, a major limitation in proposing microdomains is the measurement of processes in microdomains – or to identify selective targeting within a cell. Current developments in signalling are telling us how probably oversimplistic our views of the cell can be. The picture that is developing – in the case of Ca\(^{2+}\)-sensitive ACs, at least – of a discrete domain involving ACs in lipid rafts, held there by protein–protein interactions with other raft residents, along with CCE channel components, NHE1, gravin, PKA and PDE4D, and in which cAMP kinetics differ dramatically from those that reach the cytosol, and which therefore may evoke distinct signalling consequences, is likely to be just a preview of the true complexity that is cAMP signalling. We can expect to fill in more details as further probes are developed.

References


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