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Molecules of Interest

Cyclic nucleotides

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Abstract

The natural occurrence of cyclic nucleotides in higher plants, formerly a topic of fierce debate, is now established, as is the presence of nucleotidyl cyclases and cyclic nucleotide phosphodiesterases capable of their synthesis and breakdown. Here we describe the significant properties of cyclic nucleotides, also outlining their second messenger functions and the history of plant cyclic nucleotide research over its first three decades. Findings of the last five years are detailed within the context of the functional role of cyclic nucleotides in higher plants, with particular emphasis upon nucleotidyl cyclases and cyclic nucleotide-responsive protein kinases, -binding proteins and –gated ion channels, with future objectives and strategies discussed.

Keywords: Review; Cell-signalling; Cyclic AMP; Cyclic GMP; Cyclic nucleotides; Nucleotidyl cyclases; Cyclic nucleotide-gated ion channels; Cyclic nucleotide-responsive protein kinases; Phosphodiesterases

1. Introduction

Nearly 50 years ago the appreciation of metabolic regulation was radically altered by the discovery of a single molecule, adenosine 3′,5′-cyclic monophosphate (cyclic AMP; cAMP). From this initial discovery the concepts of second messengers in biological signal transduction and of the role of kinases and protein phosphorylation/ dephosphorylation in regulating protein activities have evolved.

The discovery of cyclic AMP arose from studies of the effects of the hormones adrenaline (epinephrine) and glucagon upon glycogen phosphorylase activity in dog liver (Rall et al., 1957). A so-called "heat-stable" factor, produced in response to stimulation by the hormones, was isolated and found to contain equimolecular components of adenine, ribose and phosphate: at first it was deduced to be a dinucleotide, ironically the true structure of cyclic AMP was initially rejected due to the fact that the molecular models available in the 1950s were incapable of being bent to produce a 3′,5′-cyclic phosphate ring! It was one of those fortuitous coincidences that often prove to be of immense importance in scientific discovery that led to the correct structural elucidation of the heat-stable factor: concomitant with Sutherland's biochemical/physiological group seeking assistance from an eminent chemist (Heppel) of the era, another chemist (Lipkin) submitted an identical compound for structural determination, this sample being the product of the chemical alkaline hydrolysis of ATP. Eventually the structures were both determined to be adenosine 3′,5′-cyclic monophosphate. Soon afterwards a second cyclic nucleotide, guanosine 3′,5′-cyclic monophosphate (cyclic GMP; cGMP), was isolated from living tissues and subsequently found to carry out second messenger roles, and other cyclic nucleotides, for example cytidine 3′,5′-cyclic monophosphate (cyclic

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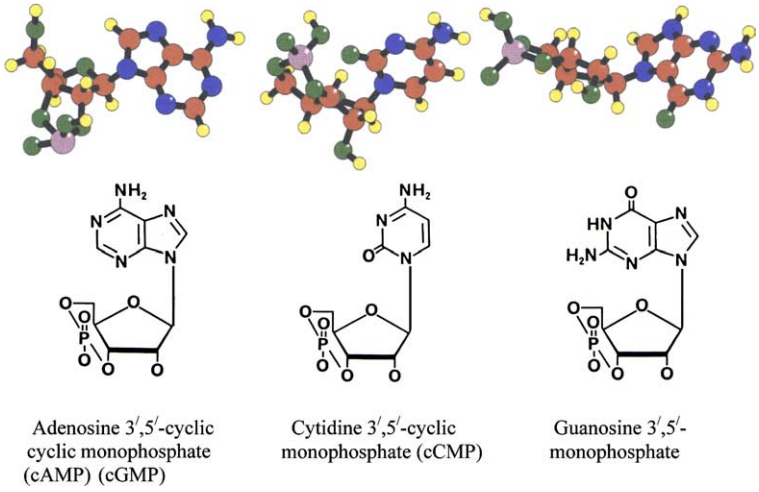

Fig. 1. Structures and conformations of cyclic nucleotides.

CMP; cCMP) (Fig. 1), have also been demonstrated to be naturally occurring.

The molecular conformations of the cyclic nucleotides are key factors in determining their chemical properties and biological activities (in addition to rendering them unrepresentable by old-style molecular models!). These conformations have been established by X-ray crystallography and proton-NMR; the 3′,5′-*trans*-fused phosphate of the riboside 3′,5′-cyclic phosphates restricts the conformation of the furanose ring, locking it into a half-chair, while the phosphate ring is locked into a chair (Ts'O, 1974; Sundralingham, 1975). This rigid conformation, particularly of the 3′,5′-cyclic phosphodiester, leads to appreciable strain, which is why the chemical synthesis of cyclic nucleotides requires fairly drastic conditions and their biosyntheses are extremely endergonic. Paradoxically the cyclic nucleotides are much more stable to acid and alkaline hydrolysis than their phosphomonoester counterparts; of more relevance from our biochemical perspective, the cyclic nucleotides are smaller and less polar molecules thus allowing their ready differentiation from non-cyclic nucleotides, for example by cyclic nucleotide-responsive protein kinases and other cyclic nucleotide-binding proteins. While the glycosidic bond between the ribose and heterocyclic rings can theoretically freely rotate, conformational energy calculations indicate that cyclic GMP favours the *syn* to the *anti* conformation 13:1, cyclic AMP favours *anti* to *syn* 2:1, and cyclic CMP favours *anti* to *syn* 99:1 (Yathindra and Sundralingham, 1974), indicating that specificity for cyclic nucleotides may result not only from the identity of the base, but also by its relative conformation to the ribose phosphate moiety during channeling to recognition sites (Shabb and Corbin, 1992).

As the prototype second messenger, the cyclic AMP system, as originally portrayed by the 1971 Nobel Prize winner Sutherland and colleagues, was a brilliantly simple concept, in which mammalian hormones and neurotransmitters, as primary messengers, remain exterior to the cell while binding to specific receptors, which recognize them, on the exterior membrane surface, resulting in change in conformation of the receptor. This change transmits a signal that stimulates adenylyl cyclase on the interior of the cell membrane, the activation of this enzyme resulting in the synthesis and release of the secondary messenger cyclic AMP into the cell. Inside the cell cyclic AMP stimulates a protein kinase, which in turn phosphorylates substrate proteins thereby altering their activity, before the signal is switched off by the hydrolysis of cyclic AMP to AMP by phosphodiesterase (Robison et al., 1971).

Further revelations have complicated this picture, as depicted in Fig. 2. Two populations of receptors are now know to exist, R_s, which stimulate the cyclase, and R_i, which inhibit, and while the primary messenger binds to the receptor on the outside of the membrane, as *per* the original concept, the receptors are actually of a characteristic serpentine or seven-pass structure, that crosses the membrane seven times (Strader et al., 1994). Located between the receptors and adenylyl cyclase are G-proteins, heterotrimeric GTP-binding proteins, which undergo a sequence of events of binding GTP, dissociating into α and β_γ subunits, interacting with the cyclase, losing a phosphate group converting GTP to GDP, reassociation and loss of interaction with the cyclase, then loss of GDP, followed by binding of GTP and initiation of a new cycle. The rate of this cycle is increased by interaction with a receptor that has bound the primary messenger (Gilman, 1987). The adenylyl cyclase unit, while having a catalytic site on the inner side of the plasma membrane crosses the membrane six times, again in contrast to the original concept,

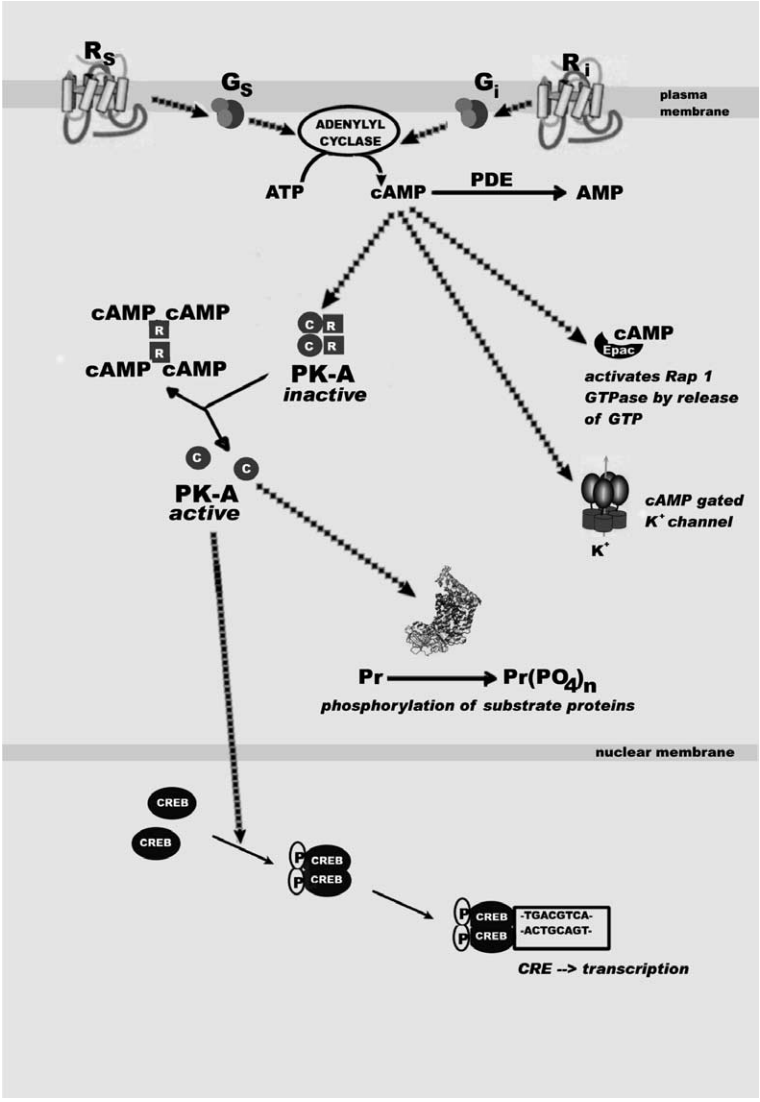

Fig. 2. Cyclic AMP second messenger system in mammalian cells.

and exists in at least nine isoforms. While being responsive not only to hormones and neurotransmitters, via the stimulatory and inhibitory G-proteins, the cyclase is also sensitive to Ca^{2+}, aluminium fluoride and forskolin, to cholera and other toxins which exert their effects by ADP-ribosylation, and to phosphorylation (Tang and Hurley, 1998). The phosphodiesterase that hydrolyses cyclic AMP and acts as the signal "off-switch" is not a single enzyme but represents many isoforms from several families of phosphodiesterases, which differ significantly in distribution, specificity and kinetics and are pharmacological targets for selective inhibitors ranging from caffeine to ®Viagra! (Beavo and Houslay, 1990).

According to the original second messenger concept, when released into the cell cyclic AMP elicited all its responses by activating cyclic AMP-dependent protein kinase (protein kinase A; PKA; A-PK) and initiating changes in the activity of extra-nuclear proteins by phosphorylating them. Two isoforms exist of PKA, both of which are dimers; on recognition of cyclic AMP PKA dissociates to yield a regulatory dimer, composed of two subunits each binding two molecules of cyclic AMP, and two free catalytic units, which in this free form are active, but are inactive when associated with the regulatory units in the absence of cyclic AMP (Francis and Corbin, 1994). The varied substrate proteins, on phosphorylation, change conformation as a result of increased surface charge, thereby altering (usually increasing but not always) their activity, a process that can be reversed by a group of specific phosphatase enzymes. While this process is traditionally regarded as a short-term, sudden effect, mechanism of fine control, it is also now recognised as a means of regulation of gene

expression, i.e., coarse control. Migration of the active PKA catalytic unit to the nucleus allows phosphorylation of the transcription factor, cAMP-response element binding protein (CREB); on phosphorylation CREB dimerizes and in this state is able to interact with DNA at the cAMP response element (CRE), an 8-base pair palindrome, allowing initiation of transcription in the presence of two other proteins, CPB (CREB binding protein) and p300, both large proteins which only interact with CREB as its phosphorylated dimer (Cesare et al., 1999).

Further direct actions of cAMP that do not involve protein kinase activation have now been identified; in odorant cells, a cAMP-gated K^+ channel, made up of four proteins assembled in such a way that the membrane-spanning domains combine to form a central pore, is a key component in a signalling pathway where odorants bind to receptors linked to another form of G-protein, G_{olf}, which activates adenylyl cyclase (Bradley et al., 1994). A second direct effect of cyclic AMP involves Rap 1, a GTPase located in organelle membranes, that functions in cell proliferation, differentiation and morphogenesis; Rap 1 activation involves binding to Epac (Exchange protein directly activated by cyclic AMP), after the latter's activation by cAMP, Epac being a guanine nucleotide exchanger facilitating the release of GDP from Rap 1 and its replacement with GTP (De Rooij et al., 1998).

In mammals at least, cyclic AMP mediates the action of a wide range of hormones and neurotransmitters and has been quoted as capable of regulating at least one enzyme in every known mammalian metabolic pathway. In contrast, the second cyclic nucleotide to be established as a metabolic regulator, guanosine 3′, 5′-cyclic monophosphate (cyclic GMP), has a much more restricted role, for example (a) in response to light activation of the visual pigment, an activated G-protein interacts with cyclic GMP phosphodiesterase, the resultant change in cyclic GMP levels altering the permeability of the eye rod cell membrane to sodium ions which in turn initiates a transmission along the optic nerve (Stryer, 1986); (b) mediating the contraction/relaxation cycle in smooth muscle in response to nitric oxide (Moncada et al., 1992); (c) changes in cyclic GMP in response to guanylin and natriuretic peptides regulate the movement of sodium ions and water across membranes (Hofmann et al., 1992). Although there are very clear differences in the physiological effects of the two nucleotides, enzymes for cyclic GMP metabolism analogous to those for cyclic AMP are present, namely guanylyl cyclase, cyclic GMP-dependent protein kinase and cyclic GMP phosphodiesterases. In mammals four other cyclic nucleotides, cyclic-CMP, -IMP, -dTMP and UMP have been demonstrated to occur, together with enzymes capable of their synthesis and hydrolysis (Newton et al., 1984a,b, 1986): their function has not been elucidated although initial evidence suggests that their varied effects are not solely due to action as cyclic-AMP or -GMP agonists or antagonists (Brus et al., 1984), with cyclic CMP effects and fluctuations in concentration compatible with a role in regulation of cell proliferation (Newton, 1995).

Because of the multiplicity of actual and potential pharmacological applications, most of the research effort into cyclic nucleotide biochemistry has been concentrated upon mammalian systems. However while their actions are well elucidated in the complex mammalian eukaryotic cell, cyclic nucleotides are also shown to occur in the simplest prokaryotes, the Eubacteria and Archae (Botsford and Harman, 1998). Their function here seems very different to the eukaryotic function, but nevertheless key regulatory functions occur and in lower organisms can be loosely interpreted as part of a response system to starvation. In *Escherichia coli* cAMP is synthesized in response to reduced glucose levels and induces expression of other nutrient degradative enzymes such as β-galactosidase, in yeast cAMP is a growth signal synthesized in response to nutrients, while in the slime mould *Dictyostelium* both cAMP and cGMP are involved in regulating the process assembling individuals cells into a multicellular slug. Given cyclic nucleotide signalling functions in organisms ranging from the prokaryotes to the primates, what do we know of their biochemistry in higher plants?

2. Cyclic nucleotides in higher plants

2.1. History

Reports of the occurrence, and theories of the functions, of cyclic nucleotides in higher plants were fiercely debated over an extended period of almost three decades. As discussed above cyclic nucleotides had been shown to be ubiquitous and have second messenger signalling functions in animals, and to perform parallel regulatory functions in bacteria and lower organisms; publications suggesting analogous roles in higher plants in the 1970s and early 1980s were criticized on the basis of:

(a) effects elicited by cyclic nucleotides lacked specificity,
(b) the identification of endogenous putative cyclic nucleotides was equivocal,
(c) the identification of cyclic nucleotide-related enzymes was ambiguous.

Perseverance by the believers has now prevailed, with the advent of more rigorous identification procedures enabling the irrefutable demonstration of the presence of cyclic nucleotides, nucleotidyl cyclases and cyclic

nucleotide phosphodiesterases, and in identifying plant processes subject to cyclic nucleotide regulation.

Numerous reviews reflect the gradual change in stance of the concensus of opinion regarding cyclic nucleotides in higher plants: initially reviews discounted the reports of the occurrence of cyclic AMP in plants, *inter alia* "failure to cyclic adenosine 3′,5′-monophosphate" (Niles and Mount, 1973), "evidence against the occurrence of cyclic AMP in higher plants" (Amrhein, 1974), and "attempts to detect cyclic adenosine 3′,5′-monophosphate in higher plants by three assay methods" (Bressan et al., 1976), with other influential reviewers of the period also concluding that cyclic AMP was not present in higher plants (Keates, 1973; Lin, 1974; Amrhein, 1977). Such negative interpretations were replaced by a more optimistic appraisal of the available evidence (Brown and Newton, 1981; Newton and Brown, 1986), and following the unambiguous mass spectrometric demonstration of the identity of extracted putative cyclic nucleotides (Newton et al., 1980, 1984a,b, 1989,; Newton, 1996), there have been few dissenting voices over the presence and potential functionality of cyclic nucleotides in plants (Spiteri et al., 1989), but instead the occurrence is agreed and discussion centres upon the detailed physiological functions (Assmann, 1995; Bolwell, 1995; Trewavas, 1997; Newton et al., 1999a,b). For a detailed description of cyclic nucleotides in higher plants research in the period 1969–1999 the reader is directed to the relevant Tansley Review (Newton et al., 1999a,b); here the latter era is summarized and then post-1999 developments elaborated in ensuing sections.

Inconsistency in data relating to cyclic nucleotide concentrations (significantly lower than those in mammals) was overcome by recognition of the inadequacy of methodologies designed primarily for use with animal extracts, devoid for example of the complexity of plant secondary metabolites (Brown and Newton, 1992). Not only has mass spectrometry been crucial in unequivocally establishing the identity of putative cyclic nucleotides extracted from plant tissues, hyphenated liquid chromatography and electrospray mass spectrometry has provided an excellent means of reliably and reproducibly quantitating cyclic nucleotides as low as 25 femtomoles in plant extracts and incubations (Witters et al., 1996, 1997a,b, 1998; Ehsan et al., 1998). Such methodology now allows the investigation of cyclic nucleotide concentrations in plant systems with complete confidence.

Adenylyl cyclase activity was initially reported in higher plant material after application of both histochemical and biochemical procedures, but while these first such reports were heavily criticised due to inadequate identification of the newly formed putative cyclic nucleotide product, more rigorous separatory procedures provided more credible evidence, then by using mass spectrometric techniques unambiguous identification of the reaction product of adenylyl cyclase activity in *Pisum sativum* was provided for the first time (Pacini et al., 1993). cAMP formation was unequivocally confirmed by mass spectrometric analysis of adenylyl cyclase activity in *Medicago sativa* cell cultures exposed to the elicitor of the phytopathogenic fungus *Verticillium albo-atrum* (Cooke et al., 1994a,b), and in plasma membrane preparations from *Phaseolus vulgaris* apical hooks (Roef et al., 1996; Roef, 1997), this association with plant plasma membrane preparations appearing compatible with a mammalian type second messenger system. The missing link was the absence of a plant gene sequence with high homology to that of mammalian adenylyl cyclase; while one such report was made in 1997 (Ichikawa et al., 1997) it was however later withdrawn (Ichikawa et al., 1998), in circumstances described by Balter (1999) and as yet the application of molecular biological techniques has failed to match the progress made by biochemical and mass spectrometric studies.

Plant phosphodiesterase studies faced a different problem to that of extracted cyclic nucleotide and cyclase product identification: a phosphodiesterase, capable of hydrolysing cyclic AMP to AMP was reported in pea seedlings (Liberman and Kunishi, 1969) before the first reports of cyclic nucleotide-containing extracts from higher plants. However Lin and Varner (1972) reported that unlike its mammalian counterpart the phosphodiesterase from pea seedlings had an acidic pH optimum, was insensitive to methylxanthines, yielded 3′-AMP rather than 5′-AMP as the major hydrolytic product, and, most significantly of all, had substantially greater activity with the RNA breakdown intermediate 2′,3′-cyclic AMP as substrate compared to that with the putative second messenger isomer, 3′,5′-cyclic AMP. However further examination of more purified plant phosphodiesterases indicated that more than one form is present. Seedlings of *Phaseolus* coccineus (Dwarf French bean) were found to contain a phosphodiesterase which, when partially purified, possessed properties more similar to those of the mammalian phosphodiesterases (Brown et al., 1975, 1977). In *Spinacea oleracea* three forms of phosphodiesterase were observed (Brown et al., 1979); while one conformed to the profile described by Lin and Varner (1972), a second isoform had highest activity with 3′,5′-cyclic GMP and 3′,5′-cyclic AMP and little activity with their 2′,3′-isomers, displayed sensitivity to endogenous protein effectors and was activated by Ca^{2+} (Brown et al., 1979; Dupon et al., 1987), occurring in multienzyme complexes in association with acid phosphatase, ribonuclease, nucleotidase and ATPase (Brown et al., 1980). A plurality of phosphodiesterases has also been reported in other species including *Solanum tuberosum* (Ashton and Polya, 1975), *Portuluca* (Endress, 1979) and *Daucus*

carota (Kurosaki and Kaburaki, 1995). The existence of several phosphodiesterase isoforms offers a ready explanation of the apparent incompatibility of the data obtained and conclusions drawn by different groups in the earlier reports of plant phosphodiesterases, with different extraction and purification protocols selecting for one or other of the phosphodiesterase types, while the presence of phosphodiesterase in a complex that also contains nucleotidase suggests that identification of one or other mononucleotide isomer as the major product of phosphodiesterase activity may not be as clear-cut as it first appeared. A further complication is the phosphodiesterase extracted from *Lactuca sativa* (Chiatante et al., 1986, 1987, 1988, 1990) which differs from other plant 3′,5′-cyclic nucleotide phosphodiesterases in that it exhibits comparable activity with both pyrimidine and purine cyclic nucleotide substrates, showing significant similarity to the multifunctional phosphodiesterase isolated initially from pig liver (Helfman et al., 1981).

Collectively the data described above demonstrated the natural occurrence of cyclic nucleotides in higher plants together with the presence of enzymes capable of the specific synthesis (nucleotidyl cyclases) and hydrolysis (cyclic nucleotide phosphodiesterases) of cyclic nucleotides. Thus plants were shown to contain the on and off switches for cyclic nucleotide second messenger systems, so the search for cellular targets for cyclic AMP action followed.

There are no reports of a plant cyclic AMP-dependent protein kinase being purified to homogeneity; however three cyclic nucleotide-responsive protein kinases have been reported in *Lemna paucicostata* (Kato et al., 1983), a cyclic AMP-dependent protein kinase was also shown in *Zea mays* and in *Cocos nucifera* (Janistyn, 1988, 1989), and a cAMP-responsive protein kinase activity in rice (Komatsu and Hirano, 1993) and *Petunia* (Polya et al., 1991). Molecular biological evidence was put forward for the presence of protein kinases with high homology to cyclic nucleotide-dependent protein kinases from other organisms in higher plants, including *P. vulgaris* and *Oryza sativa* (Lawton et al., 1989), *Z. mays* (Biermann et al., 1990), *Pisum sativum* (Lin et al., 1991) and *Arabidopsis thaliana* (Hayashida et al., 1992, 1993; Mizoguchi et al., 1992). By analogy with the mammalian CREB system, the potential of cyclic AMP regulating plant physiological processes by altering gene expression was first proposed by Inamdar et al. (1991), who went on to isolate a cDNA clone from *Vicia faba* with close resemblance to the animal CREB-protein (Ehrlich et al., 1992).

While a large number of physiological processes in plants are potentially sensitive to alterations of cyclic AMP level (see reviews, Brown and Newton, 1981; Newton and Brown, 1986; Assmann, 1995; Bolwell, 1995), whether most of these observations are valid in vivo is questionable. By the 1990s there were however more reliable indications of cyclic AMP functions in several plant processes, for example a role in ion transport in *A. thaliana* (Anderson et al., 1992), in cell cycle progression in tobacco BY-2 cells (Ehsan et al., 1998), and in the plant defence response which produces phytoalexins (Kurosaki et al., 1987; Kurosaki and Nishi, 1993a,b; Smith, 1996; Kurosaki, 1997), the latter involving a cAMP activation of phenylalanine ammonia lyase (Bolwell, 1992; Cooke et al., 1994a,b).

In the 1990s there was strong evidence produced that cyclic GMP is involved in the action of phytochrome (Bowler et al., 1994; Neuhaus et al., 1993, 1997), has a role in ion channel regulation, (Anderson et al., 1992; Sentenac et al., 1992; Hoshi, 1995; Schuurink et al., 1998), and is an important early compound in the response of the cereal aleurone to gibberellic acid (Penson et al., 1996a,b, 1997). An analogy with the mammalian system was the response to nitric oxide, with nitric oxide stimulating cGMP formation in spruce (Pfeiffer et al., 1994), a stimulation believed to be a key response in plant cellular defence driving activation of phenylalanine ammonia lyase (Durner et al., 1998).

The occurrence of cyclic nucleotides other than cyclic AMP and cyclic GMP, cyclic-CMP, -UMP, -IMP and dTMP, in *Pisum* roots was also established by MS (Newton et al., 1989). The relative levels in meristematic and non-meristematic tissues showed significant differences, with the greater quantity of cyclic CMP in the meristem hypothesized to reflect a role in the rapidly dividing cells, analogous to that posed for cyclic CMP in mammalian cells.

3. Recent developments

3.1. Nucleotidyl cyclases

In the absence of any as yet undetermined alternative pathway of biosynthesis, the presence of cyclic nucleotides in plant tissues means that nucleotidyl cyclases must also be present. This deduction is supported by the observed effects of various agonists and antagonists of mammalian cyclase activity on the accumulation of the cyclic nucleotide in plant tissues and/or the various associated plant physiological responses; for example, pollen tube growth (Moutinho et al., 2001; Tsuruhara and Tezuka, 2001), stomatal regulation (Cousson, 2003; Cousson and Vavasseur, 1998; Jin and Wu, 1999; Pharmawati et al., 1998, 2001), cell cycle progression (Ehsan et al., 1999), gene expression in aleurone of *Hordeum vulgare* (Penson et al., 1996a,b), root development (Cousson, 2004; Pagnussat et al., 2003), phytoalexin accumulation (Zhao et al., 2004) and regulation of flowering (Szmidt-Jaworska et al., 2004).

However, in addition to the earlier MS-based demonstrations of plant nucleotidyl cyclases described above,

there have also been further direct demonstrations of cyclase activity. Mass spectrometry has now been used to characterise nucleotidyl cyclase activity associated with chloroplasts from *S. oleracea* (Newton et al., 1999a,b) and *Nicotiana tabacum* (Witters et al., 2004). The activities from spinach were located in a membrane preparation and kinetic analyses indicated the presence of two cyclase enzymes. One, with guanylyl cyclase activity, showed a very high specificity for the substrate, while the other, the adenylyl cyclase, was stimulated by the GTP analogue, guanylyl phosphoimidoposphate (GMPPNP), suggesting interaction of the cyclase with a G-protein. The adenylyl cyclase in chloroplasts of tobacco also appears to be G-protein mediated since guanosine 5′-*O*-(2-thiodiphosphate)($GDP_{\beta}S$) inhibited activity whilst GTP and its analogue guanosine 5′-*O*-(3-thiotriphosphate) ($GTP_{\gamma}S$) was stimulatory (Witters et al., 2004). It is of some interest, therefore, that GTP-binding proteins appear to play a part in cell death (Kawasaki et al., 1999), the oxidative burst in infected plant cells (Kieffer et al., 1997; Bindschedler et al., 2001), induction of elicitor-induced phytoalexin synthesis in carrot cells (Kurosaki et al., 2001), and auxin-induced stomatal opening (Cousson and Vavasseur, 1998), all responses that also involve cyclic nucleotides. The same elicitor used in the carrot cell study also induces an increase in the intracellular concentration of cyclic AMP, suggesting that the target of the elicitor is a G-protein-mediated adenylyl cyclase (Kurosaki and Nishi, 1993a,b). Induction of the apoplastic oxidative burst in cultured French beans cells by the G-protein activator, cholera toxin, and the adenylyl cyclase activator forskolin suggesting the presence of a G-protein-mediated adenylyl cyclase (Bindschedler et al., 2001). In common with adenylyl cyclases from other kingdoms, the chloroplast enzyme was inhibited by alloxan and dideoxyadenosine, however, forskolin, an activator of mammalian adenylyl cyclases, had no effect.

Association of cyclase activity with chloroplasts is interesting; the concentrations of cyclic AMP in three macroalgae appear to be regulated by light (Gordillo et al., 2004), the effect being related to photosynthetic activity rather than activity of phytochrome. So, within the chloroplast, cyclic AMP may link the photochemical reactions to other functions, for example export of photosynthate. In cyanobacteria, cyclic AMP concentrations change rapidly in response to environmental changes, and in *Anabaena cylindrica* cyclic AMP acts as a second messenger in light signal transduction (Ohmori et al., 2002). In seedlings of *Avena sativa*, however, the activity of adenylyl cyclase does appear to involve phytochrome, since red light decreased the activity of a membrane bound adenylyl cyclase whilst far red light reversed this effect (Molchan et al., 2000). Activation by GTP suggests that the activity of this cyclase is also mediated by interaction with a G-protein.

Until recently, such evidence of cyclase activity has been treated with some scepticism, because of the failure to identify DNA sequences in plant genomes with significant homology to those of established cyclases. However, there are now reports of cloning of putative cyclases. One from *Z. mays*, PsiP (pollen-signalling protein), represents an adenylyl cyclase (Moutinho et al., 2001), while the other from *A. thaliana* shows three times greater guanylyl than adenylyl cyclase activity (Ludidi and Gehring, 2003). In both cases, recognising the diversity of known adenylyl cyclases, candidate proteins for functional testing were identified by searching the databases for sequences with homology to adenylyl cyclases other than the ''classic'' mammalian type enzymes. In fact, PSiP shows homology not only to fungal adenylyl cyclases (*Neurospora crassa*) but also to disease-resistance proteins (Moutinho et al., 2001; Talke et al., 2003), which may be of some significance given that cyclic nucleotides appear to play a role in pathogen response signalling (Cooke et al., 1994a,b; Durner et al., 1998; Zhao et al., 2004). The cyclase from *Arabidopsis*, *AtGC1*, was identified by querying the *Arabidopsis* genome with a motif based on conserved and functionally assigned amino acids in the catalytic domain of annotated GCs from vertebrates, lower eukaryotes and prokaryotes. Both proteins were expressed in *E. coli*, resulting in significant increases in the intracellular content of the relevant cyclic nucleotide in the bacterial cell, and the sequences of both proteins indicate they are soluble type cyclases. *AtGC1* shows no significant sequence similarity with the haeme-binding domain that interacts with NO and which is present in other guanylyl cyclases, and indeed the recombinant guanylyl cyclase activity was insensitive to the NO donor, nitroprusside. While the function of the guanylyl cyclase from *Arabidopsis* is not yet known, the adenylyl cyclase activity deduced to be present in *Agapanthus* appears to modulate cyclic AMP concentration (Moutinho et al., 2001) and in doing so is critical in maintaining transduction mechanisms that operate during pollen tube growth (Moutinho et al., 2001; Tsuruhara and Tezuka, 2001).

A cDNA encoding an adenylyl cyclase-associated protein (CAP) has been isolated from *Gossypium hirsutum* (Kawai et al., 1998). In *Saccharomyces cerevisiae* CAP is a binding partner of adenylyl cyclase, which is activated by the G-protein Ras (Shima et al., 1997). In general, CAPs regulate actin remodelling in response to cellular signals, the N terminus playing a role in Ras signalling and binding adenylyl cyclase. Expression of the CAP gene was highest in young fibres and it appears that the gene may play a role in cell elongation in this tissue, possibly through effects on the cytoskeleton. It will be interesting to determine whether such a gene is expressed in the pollen tubes of *Agapanthus*.

3.2. Cyclic nucleotide-responsive kinases and binding proteins

There is little recent direct biochemical evidence of cyclic AMP-dependent protein kinase (PKA) activity in plants, though demonstrations of cyclic AMP-dependent phosphorylation in etioplasts and chloroplasts of wheat (Friedrich et al., 1999), and in a cell-free system derived from guard cells of *V. faba* L. (Sharma et al., 1999) imply that such activity exists. While the millimolar concentration of cyclic AMP used in the study with wheat tissues is clearly not physiological, micromolar cyclic AMP was used in the French bean study, and forskolin increased phosphorylation of those same polypeptides that were phosphorylated in response to exogenous cyclic AMP. Furthermore, inhibitors of PKA activity inhibited the forskolin-induced enhancement of protein phosphorylation (Friedrich et al., 1999). However, in neither of these studies were the kinase activities isolated.

The PKA inhibitor, K252a, partially inhibited abscisic acid (ABA) induction of β-glucuronidase (GUS) activity in tobacco cells transformed with a ABA-responsive promoter (*rd29A*)-*GUS* reporter construct (*rd29A-GUS*) (Liu et al., 2002). Interestingly, ABA induces PKA activity (PKABA1) that inhibits induction of amylase synthesis in barley aleurone (Gomez-Cadenas et al., 2001).

Protein kinase G (PKG) inhibitors prevented auxin-induced stomatal opening implying the presence of PKG in *Commelina communis* (Cousson and Vavasseur, 1998). However, a cyclic GMP-regulated protein kinase has now been purified from tissues of *Pharbitis nil* with a combination of anion-exchange- and affinity-chromatography (Szmidt et al., 2003). The enzyme is a polypeptide of M_r 70 kDa, its activity with a mixture of histones as substrate was stimulated by cyclic GMP at micromolar concentrations, and it cross reacted with antibodies raised against the catalytic domain of a PKG from guinea pig. There was also evidence of autophosphorylation on both serine and threonine residues that was stimulated by cyclic GMP, which is a characteristic of the PKG from mammalian cells.

Cyclic GMP-binding activity has been detected in a protein fraction from *A. sativa* (Dubovskaya et al., 2002). Two binding sites (one with high- the other with low affinity to cyclic GMP) were associated with a protein from a soluble fraction, and binding was increased by red light and by Ca^{2+}. No information is available concerning the identity of the protein. In contrast, the cyclic AMP-binding activity that was detected in a soluble protein fraction of tobacco bright yellow 2 cells was associated with three proteins; glyceraldehyde 3-phosphate dehydrogenase and two nucleoside diphosphate kinases (NDPK) (Laukens et al., 2001). On the basis of NDPK function in yeast and animals, and their potential to act as a transcription factor in the response to UV in plants (Zimmerman et al., 1999), the authors suggest a function for the NDPK isoforms in signal transduction, possibly in cell cycle progression.

There have now been reports of protein sequences in plants with characteristics of the cyclic nucleotide-dependent protein kinases present in yeast and animal cells (Liu et al., 1999; Hammond and Zhao, 2000; Vallad et al., 2001). A gene, *SPBK*, from germinating pollen of *Solanum berthaultii* encodes a protein that shows closest similarity to the cyclic AMP-or cyclic GMP-dependent protein kinases, a group of serine/threonine protein kinases (Liu et al., 1999). *SBPK* does not possess any potential regulatory domains, indicating that *SBPK* probably exerts its function in combination with other, regulatory, subunits in a manner similar to many cyclic AMP-dependent protein kinases in animals. Inactivation of endogenous *SBPK* expression (through expression of antisense *SBPK*) resulted in plants producing a high proportion of aberrant pollen grains. Bearing both this and the evidence that cyclic AMP may regulate growth of pollen tubes (Moutinho et al., 2001; Tsuruhara and Tezuka, 2001) in mind, a role for this protein in signal transduction during development is indicated, although there is not yet direct evidence.

In *Lycopersicum esculentum* the gene *pkv*, transcriptionally activated in plants by a severe strain of the potato spindle tuber viroid, encodes a protein kinase with significant homologies to cyclic-dependent protein kinases, (Hammond and Zhao, 2000). *PKV* also has a putative sequence for nuclear targeting of proteins, which may target *PKV* to the nucleus. The significance of such targeting is not known, though one potential result of such migration could be interaction with a CREB. Interestingly, the promoter region of the *PKV* gene contains a G-box element that appears to function in regulation of genes by developmental stimuli such as abscisic acid, pathogens and light, all responses in which cyclic AMP has been implicated (Jin and Wu, 1999; Molchan et al., 2000; Liu et al., 2002; Zhao et al., 2004).

Very recently exciting new evidence has been produced: plant protein kinase sequences have been discovered that carry putative cyclic nucleotide binding domains. Full length cDNA's of putative cyclic nucleotide binding proteins have been isolated from *A. thaliana* and *N. tabacum*, carrying both cyclic nucleotide binding, protein kinase and even protein phosphatase signatures (Roef, unpublished observations, personal communication). Blast searches reveal the existence of several EST's from *A. thaliana*, *L. esculentum*, *Medicago truncatula*, rice, wheat and others, all carrying similar cyclic nucleotide binding domains. Expression analysis hints at roles in cell cycle, light, stress and/or hormone responses (Roef, unpublished observations, personal communication).

3.3. Cyclic nucleotide phosphodiesterases

The evidence of cyclic nucleotide specific phosphodiesterase activity in plants is well established (see above). However there are comparatively far fewer recent studies, though the biophysical characteristics of a phosphodiesterase from *Arabidopsis* have been determined (Hofmann et al., 2002). Mass spectrometry was used to examine the effects of various cations on a phosphodiesterase complex from chloroplasts of *P. vulgaris*, indicating that the preferred substrate is a 3′,5′-cyclic nucleotide and that Fe^{3+} stimulates hydrolysis of cyclic CMP (Smith et al., 2001). In a study of regulation of enzyme activities by different CaM isoforms, two isoforms from *Glycine max* were shown to have different requirements for Ca^{2+} for interaction with phosphodiesterase (Lee et al., 2000). It is an interesting parallel with mammalian/pharmacological cyclic nucleotide studies that for the first twenty or twenty five years most research effort in the latter area was concentrated upon adenylyl cyclase and protein kinases as being the trigger and mechanism of action of cyclic AMP, respectively, with phosphodiesterase being regarded as an uninteresting maintenance enzyme which performed at a fairly consistent basal activity, until the more recent recognition of a large number of phosphodiesterase isoforms with significantly different isosteric and allosteric sensitivities (Beavo and Houslay, 1990).

3.4. Cyclic nucleotide-gated ion channels

While it may be difficult to establish the case that PKAs are the downstream targets and effectors in plant cyclic nucleotide signalling pathways, there is increasing evidence that the group of plant ion channels, Cyclic nucleotide-gated ion channels (CNGCs), may be candidates (Trewavas et al., 2002; Talke et al., 2003). CNGCs are a group of ion transport proteins that are present in many plant species, including *Arabidopsis*, barley, rice and tobacco, and that are only active when cyclic nucleotides are bound (Schuurink et al., 1998; Köhler et al., 1999; Arazi et al., 2000; Talke et al., 2003), making them potential targets for second messenger cyclic nucleotides. There are at least 20 genes encoding putative CNGCs in *Arabidopsis* (Talke et al., 2003).

Functional analysis of the cloned CNGC, AtCNGC2 from *Arabidopsis*, indicates that it can conduct movement of K^+ and other monovalent cations and Ca^{2+} but excludes Na^+ (Leng et al., 2002a,b). Conductance of K^+ and Ca^{2+} was dependent on the presence of cyclic AMP or cyclic GMP (Leng et al., 2002a), though a high external concentration of Ca^{2+}-inhibited K^+ movement (Leng et al., 2002a). In addition to a cyclic nucleotide-binding domain (CNBD), plant CNGCs have an overlapping calmodulin-binding domain (CaMBD) (Arazi et al., 2000), though different CNGCs may have different CaM binding affinities (Kohler and Neuhaus, 2000). Nevertheless, binding of CaM to AtCNGC2, which was Ca^{2+}-dependent, reversed activation of the channel by cyclic AMP (Hua et al., 2003). Half-maximal binding of CaM occurred at approximately 200 nM Ca^{2+}, a concentration that may be achieved during operation of signal cascades in plant cells (Evans et al., 2001). So, Ca^{2+}-dependent interaction between CaM- and cyclic nucleotide binding would be of physiological relevance, and CNGCs thus have the potential for integrating signals from the cyclic nucleotide- and Ca^{2+} intracellular signal transduction pathways. Little information is available concerning the subcellular localisation of CNGCs, though HvCBT1, from barley (Schuurink et al., 1998), and NtCBP4, from tobacco (Arazi et al., 2000), are located in the plasma membrane. Given the size of the CNGC family (above), combined with the possibility that they may be targeted to various cellular membranes and that they are differentially expressed in tissues of *Arabidopsis* (Talke et al., 2003), it seems likely that there is a significant diversity in the physiological functions of CNGCs. Indeed, null mutations in a single channel, *AtCNGC2*, lead to a specific hypersensitivity to Ca^{2+} (Chan et al., 2003), despite there being potentially 19 other CNGCs encoded in the *Arabidopsis* genome.

There are several physiological processes in plants in which both cyclic nucleotides and ion channel activity are involved, thence the activities of CNGCs may be implicated. Studies with two mutants of *Arabidopsis* that are impaired in the hypersensitive response (HR), *dnd1* and *hlm1*, have established a role in plant pathogen responses for *AtCNGC2* (Clough et al., 2000) and *AtCNGC4* (Balagué et al., 2003). Both channels are permeable to K^+ while AtCNGC2 also shows permeability to Ca^{2+}, and increases in the transmembrane fluxes of Ca^{2+} and K^+, as well as Cl^-, are some of the earliest responses to pathogens (Nürnberger and Scheel, 2001). In this regard, it is interesting to note that expression of *AtCNGC4* (Balagué et al., 2003) and an isoform of a CNGC (*PvCNGC-A*) in bean (Ali et al., 2003) were induced in response to pathogen infection. Furthermore, the expression pattern of *AtCNGC2* in *Arabidopsis* suggests a function for it in initiation of developmentally regulated cell death (Köhler et al., 2001). Both cyclic AMP and cyclic GMP are implicated in plant defence-related signalling. For example, transient increases in intracellular cyclic AMP in response to elicitors of defence responses have been observed in *M. sativa* cultures (Cooke et al., 1994a,b) and French bean cells (Bindschedler et al., 2001). Furthermore, nitric oxide (NO) treatment of tobacco leaves induces a transient increase in cyclic GMP concentration and expression of defence-related genes (Durner et al., 1998). In fact, cyclic GMP has been shown to be involved in cell death, being a required component in the signal pathway mediating NO-induced cell death in *Arabidopsis* cells (Clarke et al.,

2000). The significance of *AtCNGC2* in relation to regulation of Ca^{2+} has already been referred to (Chan et al., 2003).

Following Kurosaki's report of stimulation of K^+ flux into cultured carrot cells by cyclic AMP, accompanied by an increase in Ca^{2+} in flux (Kurosaki, 1997), there have been further reports indicating a role for cyclic AMP and/or cyclic GMP in stomatal opening, an event involving fluxes of several ions. For example, cyclic AMP reversed ABA- or Ca^{2+}-induced inhibition of both stomatal opening and whole-cell inward K^+ currents, an effect that was mimicked by the AC activator prostaglandin E_1, or the PDE inhibitor, 3-iso-butylmethylxanthine (Jin and Wu, 1999). In *C. communis* auxin-induced stomatal opening appears to involve two signal transduction pathways, one of which involves cyclic GMP as a mediator within a Ca^{2+} cascade (Cousson, 2001). A similar pathway appears to operate in *Arabidopsis* (Cousson, 2003). Stomatal opening induced by kinetin has also been shown to involve cyclic GMP and to depend on the intracellular concentration of Ca^{2+} (Pharmawati et al., 1998, 2001), while cyclic nucleotides are reported to affect fluxes of K^+, Na^+ and H^+ in *Z. mays* roots (Pharmawati et al., 1999). An interesting relationship between Ca^{2+} and cyclic GMP also appears to exist in root formation (Cousson, 2004).

An interesting relationship has been identified between cyclic AMP and Ca^{2+}, in relation to growth and orientation of the pollen tube of *Agapanthus* (Malhó et al., 2000; Moutinho et al., 2001). Dibutyryl cyclic AMP (a membrane-permeating analogue of cyclic AMP) increased intracellular Ca^{2+} in the pollen tube, while photolytic release of caged cyclic AMP caused bending of the tip, in a manner that mimics photolytic release of caged Ca^{2+}. Distribution of Ca^{2+} is an important factor in controlling tip growth and cyclic AMP appears to be an important in controlling this distribution. Cyclic AMP also appears to be involved in self-incompatibility in pollen tubes of lily (Tsuruhara and Tezuka, 2001). In another response involving cyclic nucleotides and Ca^{2+}, the intracellular Ca^{2+} concentration of tobacco protoplasts was raised by membrane-permeating analogues of cyclic nucleotides and by effectors of adenylyl cyclase and phosphodiesterase (Volotovski et al., 1998). Such results reflect earlier findings with carrot cell suspensions (Kurosaki and Nishi, 1993a,b).

Whether the channels involved in such ion movements are CNGCs is not known and will require further investigations. Neither is it clear whether cyclic nucleotides affect the activities of ion channels directly, by binding to them, or indirectly, by affecting their phosphorylation status. Certainly, cyclic AMP-dependent phosphorylation occurs in a cell-free system derived from guard cells of *V. faba* L. (Sharma et al., 1999) and phosphorylation can affect channel activity in guard cells (discussed in Jin and Wu, 1999 and Talke et al., 2003). An argument for direct regulation of channel activity by cyclic nucleotides can be found in Talke et al. (2003).

Two other examples of cyclic nucleotide regulation of channel activity are worth mentioning. Voltage-independent non-selective channels in *Arabidopsis* root protoplasts were deactivated by both cyclic AMP and cyclic GMP, which consequently played a crucial role in limiting Na^+ entry (Maathuis and Sanders, 2001). A similar inhibitory effect on Na^+ uptake has been made in *Capsicum annum* (Rubio et al., 2003). In wheat roots Al^{3+}activated an outward K^+ current only when intracellular cyclic AMP was present, the continued efflux of K^+ accompanying an efflux of malate that protects the root tips by chelating toxic Al^{3+} in the rhizosphere (Zhang et al., 2001).

3.5. Miscellaneous responses to cyclic nucleotides

Emphasis has been placed here on the enzyme systems and on the potential role of CNGCs. However, it is essential to point out that cyclic nucleotides are implicated in a number of other significant responses. For example, cyclic GMP has been implicated as part of the signal transduction pathway involving NO-mediated responses such as guard cell closure, cell death (reviewed in Neill et al., 2003) and recently, root development (Pagnussat et al., 2003). Progress in identifying the role of cyclic GMP in barley aleurone is discussed in Ritchie et al. (2002), while in wheat seeds ABA and cyclic AMP appears to promote synthesis of particular proteins, the synthesis of which is also promoted by drought (Maksyutova and Viktorova, 2003). In oat seedlings the concentration of cyclic AMP is influenced by light in a way that suggests it is part of a phytochrome signal pathway (Molchan et al., 2000). Cyclic AMP has an inhibitory effect on a phosphohydrolase activity (Tikhaya and Fedorovskaya, 2002), while cyclic AMP and cyclic GMP appear to be involved in nodulation (see for example, Terakado et al., 2003). There continues to be interest in the role of cyclic AMP in elicitation of phytoalexin synthesis (Zhao et al., 2004; Kurosaki et al., 2001).

3.6. Future directions

The energy requirement for the synthesis of cyclic nucleotides and the existence of specific enzymes responsible for their synthesis and breakdown imply that these compounds are produced for a purpose; the differences between mammalian, lower animal, prokaryote, lower plants and higher plant cyclic nucleotide system components imply that they are not merely still present in higher plants as an evolutionary anachronism. The evidence discussed above indicates that cyclic AMP and cyclic GMP at least have specific functions with parallels

to the cyclic nucleotide second messenger roles in mammals. In contrast to the paradigm of cyclic AMP actions in animal cells depicted in Fig. 2, there is no analogous simple composite figure currently possible for cyclic nucleotides in plants. One causative factor for this is the number of disparate plant species that have been examined within the context of cyclic nucleotide studies: a second is the rigour with which many such reports were appraised and dismissed in the late 1970s and early 1980s. At a workshop at the triennial International Second Messenger Congress in Brussels in 1980, one pharmacological cyclic nucleotide luminary of the time vouched the opinion that if mammalian cyclic nucleotide research had been treated with the same scepticism as it had in plants, then the field would have died soon after birth! While some of the subjective views expressed at that time may have resulted from political and non-scientific loyalties and unquestionably had a severe retarding effect upon progress in this field, the net result today is that plant cyclic nucleotide research has an ultra-solid foundation, with rigorous methodologies available for the unequivocal identification and quantitation of cyclic nucleotides and related enzymes, and the field is at last gathering exciting momentum.

The building of a complete picture of cyclic nucleotide metabolism, roles and mechanisms of action would be greatly aided by concentrated, concerted effort on elucidating the system in one or a few higher plant species. *Arabidopsis*, by virtue of the knowledge of the genome amongst other factors, would seem an obvious choice, but the tobacco BY2 culture is now similarly genetically determined (Nagata et al., 2004) and carries the additional advantage of allowing synchronous growth. Indeed we have already succeeded in demonstrating endogenous cyclic nucleotides in BY2 cells and quantitating them (Richards et al., 2002), while as described above several reports of cyclic nucleotide involvement in physiologically significant events in this species has been made. Whichever single or multiple species is selected as a model, the paradox of extensive knowledge of the biochemistry of cyclic AMP and related enzymes but little knowledge of the molecular biology, vis a vis extensive knowledge of the molecular biology of cyclic GMP-related systems but little of their biochemistry, needs to be corrected.

In the late 1960s and early 1970s when the second messenger hypothesis was being expanded, a set of criteria were generally accepted as needing to be fulfilled before a particular process could be declared to be mediated by cyclic AMP action as a second messenger. These criteria were:-

(a) the putative agonist must be shown to elevate cyclic AMP levels in whole cell preparations,
(b) the putative agonist must be shown to stimulate adenylyl cyclase in broken cell preparation,
(c) cell-permeating cyclic AMP derivatives must be able to induce the response elicited by the putative agonist,
(d) proteins that are part of the mechanism of this response must be shown to be phosphorylated in response to elevated cyclic AMP levels.

These same criteria are arguably no longer valid, particularly for plant cyclic nucleotide research. Nevertheless a concensus view of appropriate criteria would be beneficial: many molecular biologists would advocate the requirement for gene sequences of high homology to mammalian and/or bacterial nucleotidyl cyclase and cyclic nucleotide-responsive protein kinases, biochemists such as ourselves would take the more functional approach and argue that the cyclase and phosphodiesterase activities are established, as is the presence of the cyclic nucleotides, thus it is the chain from the agonist through to the end event of the cyclic nucleotide-mediated response that must be elucidated. It is our view that the application of mass spectrometric proteomic techniques (see review, Newton et al., 2004) will be the mainstay of investigations in the immediate future, facilitating identification of proteins phosphorylated and dephosphorylated in response to changes in cyclic nucleotide concentrations, identifying other qualitative and quantitative changes, including post-translational modifications, in proteins in cyclic nucleotide-mediated responses, and identifying cyclic nucleotide-binding sites.

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