Extracellular Calcium and cAMP: Second Messengers as “Third Messengers”?  

Calcium and cyclic AMP are familiar second messengers that typically become elevated inside cells on activation of cell surface receptors. This article will explore emerging evidence that transport of these signaling molecules across the plasma membrane allows them to be recycled as “third messengers,” extending their ability to convey information in a domain outside the cell.

The foundations of the second messenger concept were established nearly 50 years ago when Earl Sutherland and Ted Rall identified a heat stable factor that mediated the intracellular actions of the hormones glucagon and epinephrine on glycogen metabolism in the liver (49). That factor was, of course, cyclic AMP, a discovery that earned Sutherland a Nobel Prize in 1971 (58).

The next signaling molecule to be officially considered as a second messenger was the calcium ion (12). The importance of Ca\textsuperscript{2+} as a trigger of muscle contraction was known since the 19th century from the pioneering work of London-based physiologist Sydney Ringer. However, acceptance of the general signal transduction function of Ca\textsuperscript{2+}, as originally proposed by Lewis Victor Heilbrunn in the 1940s, initially met with resistance (43). Recognition of the validity of this theory slowly built following the identification of the Ca\textsuperscript{2+} ATPases, intracellular Ca\textsuperscript{2+} release channels, and protein targets for the Ca\textsuperscript{2+} signal throughout the 1960s and 1970s. Around this same time, several laboratories managed to make direct measurements of agonist-stimulated intracellular Ca\textsuperscript{2+} transients using the luminescent jellyfish photoprotein aequorin. Together, these findings firmly vaulted Ca\textsuperscript{2+} to the status of a bona fide second messenger.

Ca\textsuperscript{2+} and cAMP are now recognized as universal regulators of cell function. Between them, these two classic textbook second messengers impact nearly every aspect of cellular life in diverse organisms ranging from amoebae to plants to human beings. A recurrent theme that has emerged since the early descriptions of these fundamental messengers is the importance of localized signaling events within the cell (6, 16, 60, 70). By confining Ca\textsuperscript{2+} or cAMP to precise subcellular domains (e.g., plasma membrane, organelles), these molecules can selectively activate a subset of targets, thereby expanding the repertoire and range of the signal. Discovery of this property was greatly facilitated by the parallel development of fluorescent probes and digital microscopic imaging techniques, methods initially applied for the visualization of Ca\textsuperscript{2+} signaling events (71). More recently, FRET-based indicators for imaging cAMP in single cells have been used to confirm that the metabolism and disposition of cAMP can be regulated independently in different parts of the cell (25, 42, 46).

It seems that yet another domain within the organism touched by Ca\textsuperscript{2+} and cAMP is the extracellular space. It has long been known that intracellular signaling events are associated with changes in second messenger concentrations outside the cell. Can these fluctuations be regarded as “signals” in their own right? This article will address how cAMP and Ca\textsuperscript{2+} move across the cell membrane, the potential mechanisms for sensing these extracellular changes in messenger concentration, and the physiological outcomes of these signaling events.

**Export of cAMP from Stimulated Cells**

In 1963, just 5 years after the seminal descriptions of the second messenger function of CAMP, Davoren and Sutherland reported the existence of a probenecid-sensitive mechanism for cAMP extrusion in nucleated pigeon erythrocytes (18). Over the years, numerous reports have appeared demonstrating that cAMP can be expelled following agonist stimulation from a wide variety of cell types, including adipocytes (57), hepatocytes, renal epithelial cells (56), neuronal cells (53), fibroblasts, T lymphocytes (67), and skeletal muscle (27). In some earlier studies, investigators even used determinations of external [cAMP] as a surrogate for measurements of hormone-dependent cAMP accumulation inside the cell (8). Nevertheless, this ability of cells to eject the second messenger is apparently cell-type specific, since certain other cell types do not appear to extrude cAMP whatsoever.

cAMP export can proceed across a concentration gradient, is temperature dependent, and requires energy in the form of ATP (FIGURE 1). This process is susceptible to inhibitors of organic anion transport, such as probenecid and sulfipyrazone (56). In a colonic carcinoma model (CC531mdr+), the efflux of cAMP was found to be exquisitely sensitive to cell swelling (28). Certain multi-drug resistance proteins in the MRP family have now been identified as significant pathways for cAMP egress (39, 69). These proteins belong to the superfamily of ATP binding cassette (ABC) proteins, specifically the ABC subfamily. Most of these are known to function as active membrane transporters for organic anions and various drugs [e.g., for bile acids and chemotherapeutic agents, including nucleoside...
anals; see Kruh and Belinsky for review (39)]. In particular, MRP4, MRP5, and MRP8 are established transporters of cyclic nucleotides (cAMP and cGMP) (69). These drug efflux pumps often assume polarized distributions in epithelial cells (e.g., MRP4 is found in the apical membrane of kidney tubule cells and the basolateral membrane of prostate glandular cells) (39).

Quantitatively, the amount of cAMP expelled by certain cell types can be quite significant. For instance, human CD4+ T lymphocytes stimulated with cholina toxin release more than 50% of the total cAMP produced over a 24-h period to the extracellular space (67). Streifler found an even more vigorous degree of probenecid-sensitive export in polarized LLC-PK1 renal epithelial cells; 40 min after stimulation with arginine vasopressin, the extracellular cAMP concentration in the apical bath was more than twice that retained inside the cells (56). In humans, the concentration of cAMP in plasma and urine can become dramatically elevated under a variety of physiological conditions and also following infusion of endogenous cAMP-elevating hormones (particularly epinephrine, parathyroid hormone, and glucagon). For example, Hendy and colleagues showed many years ago that infusion of human subjects with a large bolus of glucagon causes massive release of cAMP from the liver, elevating plasma levels from low nanomolar levels to over 450 nM within 10 min (31). This could translate into dramatic local accumulations of cyclic nucleotides in the diffusion-restricted spaces adjacent to cells. Direct measurements using microdialysis techniques have further demonstrated that significant fluctuations in extracellular cAMP can occur in intact brain tissue during agonist treatment (11).

The cAMP extrusion mechanism has been largely dismissed as a means of regulating the intracellular cAMP signal, since the cyclic nucleotide phosphodiesterases (PDEs: the enzymes that degrade cAMP and cGMP) already provide powerful and rapid control of intracellular cAMP (3, 39). Furthermore, it has been argued that this strategy is too energetically unfavorable to be a viable means of regulating the cyclic nucleotide levels within the cell, with the further adverse consequence of depleting the cellular purine pool (38). As seen in Figure 2, however, acute inhibition of this efflux pathway with probenecid can result in measurable increases in resting intracellular [cAMP] as measured using sensitive FRET-based reporters. This suggests that in some cells this molecular device may be capable of altering resting cAMP levels and, potentially, the profile of the intracellular cAMP signal (27, 56), in addition to altering extracellular levels of cAMP.

**Actions and Targets of Extracellular cAMP**

In the social amoeba *Dictyostelium discoideum*, extracellular cAMP serves as a chemical alarm bell that signals single-celled individuals to aggregate during stress into a multicellular “slug” or pseudoplasmodium. This fascinating organism, long used as a model for cellular migration and chemotaxis, is known to express four G-protein-coupled receptors (GPCRs) used to detect secreted cAMP (cAR1, cAR2, cAR3, cAR4; not to be confused with the Ca2+-sensing receptor “CaR” described below) (5). These receptors have affinities for cAMP ranging from sub-micromolar to micromolar.

To date, no mammalian homologs of this well-characterized cAMP receptor have been reported. It is noteworthy, however, that the *Dictyostelium* GPCRs share some limited sequence similarity to the secretin family of GPCRs expressed in mammalian cells, which includes receptors for parathyroid hormone and calcitonin. It has been speculated that the latter class of GPCRs may mediate the actions of extracellular cAMP in vertebrates (5). Extracellular cAMP is known to have many actions on diverse organ...
systems, including renal, hepatic, and central nervous system. This subject has recently been reviewed in depth by Bankir et al. (5) and Jackson and Raghvendra Dubey (38). As described below, some of these actions may be the indirect result of the metabolism of cAMP to adenosine in the extracellular space (the “extracellular cAMP-adenosine pathway”), although some effects appear to be direct. For example, Sorbera and Morad (55) showed in 1991 that 50 μM extracellular cAMP rapidly (~50 ms) inhibited a sodium current in ventricular myocytes derived from several vertebrate species. This effect was sensitive to pertussis toxin, suggesting a GPCR-based mechanism dependent on Gαi or Gαo proteins (55). As another example, secreted cAMP (but not adenosine) derived from stimulated human CD4+ T lymphocytes was recently shown to exert significant growth effects on neighboring T cells in a co-culture system (67).

Detrick and colleagues demonstrated that nanomolar concentrations of extracellular cAMP and cGMP (but not adenosine or guanosine) enhanced colony formation in myeloid progenitor cells. Interestingly, membrane-permeant forms of the cyclic nucleotides used at high micromolar concentrations had the opposite effect on the proliferation of the cells, implying that an intracellular elevation of cAMP or cGMP could antagonize the action of extracellular second messenger (20). Elalamy and colleagues have provided compelling evidence for the involvement of an ecto-PKA (protein kinase A) in mediating the actions of extracellular cAMP (used at a concentration of 5 μM) on expression of prostaglandin H synthase (PGHS-2) in a pulmonary microvascular endothelial cell model (22). Again, intracellular cAMP and extracellular cAMP had opposing actions on PGHS-2 expression, causing upregulation and downregulation of the enzyme, respectively.

**Paracrine Action of cAMP on the Renal System: The Extracellular cAMP-Adenosine Pathway**

The hormone glucagon, which is released from the pancreas directly into the portal blood flow, causes intracellular cAMP signaling in hepatocytes, and, as suggested above, this leads to substantial efflux of cAMP into the general circulation. It appears that an important target of this circulating cAMP is the renal system, particularly the proximal tubule. Glucagon is well known to cause a marked enhancement of renal sodium and phosphate excretion in vivo, although specific receptors for glucagon have never been identified in the kidney. This prompted Bankir and colleagues (5) and others to propose that cAMP released from the liver might be acting as a circulating factor mediating the renal actions of glucagon. A sequence of studies by Ahloulay et al. showed that cAMP infusion alone reproduced the actions of glucagon on renal Na+ and PO4− handling (2). This phenomenon has been named the “pancreato-hepatorenal cascade.”

A comprehensive series of animal experiments carried out by Jackson and coworkers (36, 37) have given a further twist on this general theme. These investigators showed that the cAMP entering the general circulation from the liver is able to undergo enzymatic conversion to adenosine once it reaches the kidney (FIGURE 1). Adenosine has a short half-life in the circulation (~1 s); therefore, cAMP (which is stable in plasma) may be regarded as a sort of prohormone for adenosine. Once produced (either locally or at a distant site), adenosine can activate one of four different receptor subtypes (A1, A2A, A2B, and A3). Complex scenarios can be envisioned considering that adenosine receptor subtypes A1 and A3 interact with Gαi/Gαo to reduce intracellular cAMP levels in target cells, whereas the A2A and A3 subtypes serve to increase cAMP via Gαi1. Therefore, cAMP released from one cell type could conceivably initiate cAMP signaling in a neighboring cell or suppress cAMP signaling depending on the particular adenosine receptor subtype expression pattern.

As described above, the cAMP-adenosine pathway is prominent in the kidney, but substantial evidence for this phenomenon also exists in the central nervous system (11, 21, 38). Moreover, the cAMP-adenosine pathway has been speculated to be important in the cardiovascular system and also for systemic metabolic homeostasis (57). The presence of the pathway does not preclude the possibility that cAMP may exert direct actions on cells in addition to indirect effects via adenosine production.

**FIGURE 2. Blockade of cAMP extrusion with probenecid alters intracellular free cAMP**

Intracellular cAMP was imaged using a FRET-based biosensor (46) in single HEK293 cells as described previously (25). This sensor (courtesy of Dr. Kees Jalink) is based on the cAMP binding protein Epac, which has been labeled with CFP and YFP. cAMP-dependent conformational changes of the Epac protein result in changes in FRET, providing a measure of free cAMP. Acute treatment of cells with 1 mM probenecid caused a small but significant change in the resting FRET signal (the 480:535 nm emission ratio), consistent with an increase in intracellular cAMP. These data suggest that the cAMP export process can contribute to intracellular cAMP homeostasis, in addition to mediating the elevation in extracellular cAMP. Shown for comparison is the action of the direct adenylyl cyclase activator forskolin (50 μM).
**Extracellular Calcium as a Third Messenger**

Just as it has long been known that intracellular cAMP signaling events are associated with extracellular accumulation of the second messenger, so has it been long appreciated that Ca\(^{2+}\) can fluctuate outside cells, owing to activation of influx and efflux pathways for the cation during Ca\(^{2+}\) signaling events (41). As with cAMP, early measurements of hormone-stimulated Ca\(^{2+}\) signals frequently relied on determinations of Ca\(^{2+}\) in the external media. Because diffusion is greatly limited in the interstitial spaces (which occupy only a fraction of the tissue volume; e.g., ~20% in brain tissue) (66) and the buffering capacity for Ca\(^{2+}\) inside the cell is so much greater than outside, these fluxes can lead to significant alterations in free [Ca\(^{2+}\)] in the extracellular milieu.

**Fluctuations in Extracellular Ca\(^{2+}\)**

As indicated in FIGURE 3, agonist-stimulated Ca\(^{2+}\) signaling events involve 1) the release of Ca\(^{2+}\) from internal storage compartments into the cytoplasm via intracellular release channels (i.e., the InsP\(_3\) receptor); 2) the extrusion of Ca\(^{2+}\) into the extracellular space by plasma membrane Ca\(^{2+}\) ATPases (PMCA) or other export mechanisms (e.g., Na\(^+\)/Ca\(^{2+}\) exchangers); and 3) the activation of Ca\(^{2+}\) entry through store-operated channels (SOCs), such as the recently identified Ca\(^{2+}\) release-activated pathways known as the Orai proteins (47).

Tepikin and colleagues have provided direct demonstrations of the significant quantitative impact of the Ca\(^{2+}\) extrusion process on extracellular Ca\(^{2+}\) levels adjacent to stimulated cells (61–63). One study employed simultaneous real-time measurements of intracellular and extracellular [Ca\(^{2+}\)] in single pancreatic acinar cells suspended in a small droplet (40–90 times the volume of the cell; FIGURE 4). By measuring the extracellular [Ca\(^{2+}\)] in the droplet with a Ca\(^{2+}\)-sensitive dye, it was estimated that the total intracellular calcium content was reduced by 0.7 mM during cholinergic stimulation, owing to active transport of the cation by the PMCA.

Temporal and spatial separation of Ca\(^{2+}\) entry and efflux across the plasma membrane can give rise to physiologically significant excursions in extracellular [Ca\(^{2+}\)] (13, 35), particularly in polarized epithelial cells and other functionally polarized cells such as neurons (33). For example, Carropp and colleagues showed that [Ca\(^{2+}\)] in the luminal micro-compartment of the intact gastric gland increases by 200–500 \(\mu\)M following cholineric stimulation, owing to an abundance of PMCA on the apical membrane of the gastric epithelial cells (13). At the same time, a comparable depletion of Ca\(^{2+}\) was recorded in the basolateral interstitium of the intact mucosa as a result of Ca\(^{2+}\) influx via pathways located predominantly at the basal cell side. As described below, these extracellular [Ca\(^{2+}\)] fluctuations have been shown to have functional consequences.

It is also well established that specific elements of the Ca\(^{2+}\)-handling machinery (as well as certain Ca\(^{2+}\) sensors) can be confined in cell surface microdomains, such as caveolae (17, 26), potentially giving rise to local gradients of Ca\(^{2+}\) in the caveolar nanospaces. Other factors can influence free [Ca\(^{2+}\)] in the external milieu, including dilution and concentration of ionic species, owing to cellular water transport. In addition, the
transport of Ca\(^{2+}\) buffers (e.g., HCO\(_3^-\), PO\(_4^{2-}\)) would also be expected to influence the free [Ca\(^{2+}\)] in the interstitium. Ca\(^{2+}\) taken up into endocytic vesicles could conceivably impact the local extracellular [Ca\(^{2+}\)] (23). Finally, secretory vesicles are known to contain high concentrations of Ca\(^{2+}\) and other divalent cations (Zn\(^{2+}\), Mg\(^{2+}\)), and synchronous secretory activity could in principle lead to rapid increases in extracellular divalents (29, 48). Gray et al. recently proposed that liberation of these metals from vesicles of insulin-secreting cells may constitute a means of communication between cells (29) via sensors for extracellular Ca\(^{2+}\), as described in the following section.

**Extracellular Ca\(^{2+}\) Sensors**

Although specific GPCRs for cAMP have not been identified in mammalian cells, cell-surface receptors for Ca\(^{2+}\) are known to exist, and some of these have been well characterized. Without question, the best known of these is the extracellular calcium-sensing receptor (CaR), which was originally cloned from bovine parathyroid gland in 1993 by Brown and colleagues (9). The structural and functional properties of this widely expressed divalent cation receptor have been reviewed extensively elsewhere (10, 33) and will not be addressed in detail here. The CaR (of which only a single isoform appears to exist) is indispensable for life in mammals, acting as the Ca\(^{2+}\) sensor that controls systemic Ca\(^{2+}\) and Mg\(^{2+}\) homeostasis via PTH secretion. An emerging literature describes numerous physiological functions of this receptor throughout the body and in diverse vertebrate species, including birds and fish. Deletion of CaR is lethal, but the developments of viable “rescued” CaR knockout mouse models that maintain normal parathyroid function are being used increasingly to examine this receptor’s physiological role in other organ systems (1, 45).

CaR is a member of family C of the GPCR superfamily, which also includes three taste receptors (T1–T3), the GABAB receptors, eight metabotropic glutamate receptors (mGluR1–mGluR8), and six orphan receptors, including the recently characterized GPRC6A (7, 68). These receptors appear to share an evolutionary thread with CaR based on their common functional origins as nutrient/salinity sensors (15, 30). The CaR is allosterically modulated by extracellular amino acids (15). Conversely, other members of this family that are regarded as amino acid sensors, such as certain mGluRs, GABAB receptors, and GPRC6A, are modulated by extracellular Ca\(^{2+}\) (44). GPRC6A has 34% amino acid sequence identity with CaR (68) and is activated by relatively high extracellular [Ca\(^{2+}\)] (5–10 mM) (44). This receptor has been suggested to serve as a sensor for Ca\(^{2+}\) in bone (44), a tissue where local extremes in external [Ca\(^{2+}\)] are believed to occur during the bone remodeling process.

Many other cell surface proteins are susceptible to physiological fluctuations in external [Ca\(^{2+}\)] (recently reviewed in Ref. 32). These include gap-junction hemichannels (64), which can open in response to a modest (~200 \(\mu\)M) decrease in external [Ca\(^{2+}\)], and the receptor Notch, which may sense external [Ca\(^{2+}\)] to drive the establishment of right-left symmetry during embryogenesis (50, 51). A distinct Ca\(^{2+}\)-sensing receptor known as CAS has been recently described in plants (59). In addition, a number of ion channels alter their open probability depending on the local extracellular Ca\(^{2+}\), including the proton-gated cation channels ASIC1a/ASIC1b, HERG K\(^{+}\) channels, and other nonselective channels found in neuronal tissue (32).
Our laboratory demonstrated some years ago in a proof-of-concept study using a co-culture model system that it is possible for CaR to detect extracellular fluctuations in [Ca\(^{2+}\)] that occur secondary to intracellular Ca\(^{2+}\) signaling events (34, 65). This opened up the prospect that Ca\(^{2+}\) might function as a paracrine messenger, used, for example, to communicate information about the signaling status of a neighboring cell or to integrate or reinforce signals in multicellular ensembles. We further provided evidence for a variation on this theme, whereby exported Ca\(^{2+}\) can activate CaR expressed on the same cell in an autocrine fashion (19). Caroppo et al. (13) later showed a physiological role of this third messenger signaling system in the intact gastric mucosa. These investigators took advantage of information gained from their previous extracellular microelectrode studies at measuring the profile of the extracellular Ca\(^{2+}\) “signal” in the apical and basolateral microdomains following cholinergic stimulation (see above) (13). Remarkably, reproducing this physiological pattern of extracellular [Ca\(^{2+}\)] variation was able to elicit changes in pepsinogen and alkaline secretion from the tissue (14), and more recently this third messenger activity has been linked to changes in water transport (24) in the same model system. The CaR, which is expressed apically in the amphibian oxyntic cell, is involved in detecting the extracellular [Ca\(^{2+}\)] elevation that occurs in the luminal compartment of the gastric gland, although it appears that another entity may be responsible for sensing the basolateral decrease in [Ca\(^{2+}\)] (14). These intriguing data are suggestive of a novel mode of Ca\(^{2+}\) signaling that takes advantage of extracellular, rather than intracellular, changes in [Ca\(^{2+}\)], but it remains to be seen whether this process occurs in other tissues.

Other Second Messengers as “Third Messengers”?

Are there other hydrophilic signaling molecules that are exported by cells to inform neighboring cells of their signaling or metabolic status? Cyclic GMP, the second messenger generated by either atrial natriuretic peptide or nitric oxide gas via guanylate cyclases, is vigorously exported from many cell types in quantities that surpass that of cAMP (4, 54). This widespread phenomenon is mediated by many of the same MRP family members (e.g., MRP4, MRP5, MRP8) known to transport cAMP, as well as the organic anion transporter OAT1 (69). Diverse biological actions of extracellular cGMP have been described in brain and kidney [recently reviewed by Sager (54)], but as is the case for extracellular cAMP, specific molecular receptors for cGMP in mammalian cells have yet to be identified.

Isolated reports of extracellular accumulation of inositol 1,4,5 triphosphate (InsP\(_3\)) following cholinergic stimulation as measured using microdialysis techniques in brain have also appeared (40, 52). Roberts et al. found that several inositol phosphate metabolites of InsP\(_3\) appeared (in addition to InsP\(_3\)) in the interstitial space under these conditions, although it is uncertain whether the appearance of the additional inositol derivatives reflects metabolism of extracellular InsP\(_3\) or a separate transport process (52). It is not known whether InsP\(_3\) egress is a widespread phenomenon or whether it has any functional significance.

Conclusions

Second messengers are the cellular currency of information transfer. However, the generation of cAMP from ATP and the energy required to maintain the gradients that permit Ca\(^{2+}\) signaling to take place come at a certain energetic cost. Thus it is attractive to imagine that multicellular organisms might capitalize on fluctuations in extracellular second messengers to expand the informational content of the intracellular signal transduction process. The concept of the interstitial microdomain as a specialized signaling compartment is in its infancy, however. There is still much to learn about how and when the local concentrations of cAMP and Ca\(^{2+}\) change in this space and what the physiological consequences of these fluctuations are. The development of practical methods for probing the profile of such extracellular “signals” will be an important first step to understanding whether this constitutes a generalized device to extend the scope and range of second messenger molecules to a domain outside the cell. ■

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References


