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Pharmacology of intracellular signalling pathways

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This article provides a brief and somewhat personalized review of the dramatic developments that have occurred over the last 45 years in our understanding of intracellular signalling pathways associated with G-protein-coupled receptor activation. Signalling *via* cyclic AMP, the phosphoinositides and Ca^{2+} is emphasized and these systems have already been revealed as new pharmacological targets. The therapeutic benefits of most of such targets are, however, yet to be realized, but it is certain that the discipline of pharmacology needs to widen its boundaries to meet these challenges in the future.
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Abbreviations: AC, adenylate cyclase; AKAPs, kinase anchoring proteins; cADP, cyclic adenosine diphosphate; CHO, Chinese hamster ovary; DAG, diacylglycerol; eGFP, enhanced green fluorescence protein; FRET, fluorescence resonance transfer; GMP, guanosine monophosphate; GPCRs, G-protein-coupled receptors; GTPase, guanosine triphosphatase; IMPase, inositol monophosphatase; Ins(1,3,4)P_3, inositol(1,3,4)trisphosphate; Ins(1,3,4,5)P_4, inositol(1,3,4,5)tetrakisphosphate; Ins(1,4,5)P_3, inositol(1,4,5)trisphosphate; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; NAADP, nicotinic acid adenosine dinucleotide phosphate; NO, nitric oxide; PDE, phosphodiesterase; PI3Ks, phosphoinositide 3-kinases; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; PKA, protein kinase A; PLC, phospholipase C

Introduction

> Sexual intercourse began
> In nineteen sixty-three
> (which was rather late for me) –
> Between the end of the Chatterley ban
> And the Beatles' first LP.
>
> *Annus Mirabilis*, Philip Larkin

It is often difficult to decide when something started. Although 'cause and effect' has always been at the heart of pharmacology, and what happens immediately beyond the ligand–receptor interaction was, for a very long time, of secondary importance. Pharmacologists measured contraction, secretion, ionic changes and (eventually) changes in intracellular signalling intermediates, as a convenient readout of a quantifiable change in cellular activity caused by ligand-binding. This 'black box' approach to the link between cause and effect was perfectly excusable through the early period of development of pharmacology as a separate experimental scientific discipline. However, it might be argued that intracellular signalling continued to be a black box to many pharmacologists long after pioneering work had provided us with molecular insights into the key intermediates lying between cause and effect.

It was only more recently that the significance of measuring the effect of receptor-active ligands from different signalling vantage points was recognized. Hence, the advantage of assessment of effect further down a stimulus-response cascade provides better amplification and the detection of weak partial agonists. Moreover, the concept that G-protein-coupled receptors (GPCRs) can be constitutively active, and that the vast majority of antagonists possess negative efficacy, has only been revealed by assessing signalling outputs close to the receptor. Furthermore, the increasing acceptance that drugs can form ligand-specific states of the receptor and therefore 'traffic' signalling to different messenger pathways (Kenakin, 2001) has been fuelled by the ability to assess an array of signalling pathways.

The pharmacological targeting of key steps in intracellular signalling evolved rather slowly, with pharmacologists perhaps a little slow to realize that targeting intracellular, nonreceptor proteins could constitute a viable therapeutic approach. The problem of pharmacologically targeting signalling proteins downstream of cell-surface receptors was always felt to lie with specificity since our early understanding suggested much commonality between signalling pathways in different tissues or cells. More recently, molecular and biochemical approaches have revealed multiple isoforms of several signalling proteins and that signalling through identical cascades may be dramatically different due to microdomains or compartmentalization.

In particular, despite the above, I emphasize the contributions to intracellular signalling that have often emanated from Departments of Pharmacology even if they have rarely been published in the *British Journal of Pharmacology*. Perhaps this reflects the significant recruitment of 'non-card-carrying' pharmacologists into Departments of Pharmacology over the last 2–3 decades. Like many, this author came from a

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biochemical background and brought a biochemical and molecular flavour to his pharmacology. In this short review, I will focus specifically on the key intracellular signalling pathways downstream of GPCRs and some of the successful approaches adopted to pharmacologically target these proteins. Virtually all of our understanding in this area, like that of Philip Larkin's perceptions of sex, commenced in the 1960s and hence this survey covers the last 45, rather than the last 75 years.

Signalling *via* cyclic AMP

Perhaps the earliest research into signal transduction pathways focused upon the regulation of the cellular concentration of second-messenger molecules by cell-surface receptors. In 1958, Earl Sutherland & Ted Rall (Department of Pharmacology, Case Western Reserve) identified cyclic AMP as a key intermediate in the hepatic glycogenolytic response to adrenaline and glucagon, and subsequent biochemical studies quickly identified the enzymatic activities that synthesize (adenylate cyclase (AC)) and degrade (cyclic nucleotide phosphodiesterase (PDE)) cyclic AMP. Almost immediately, these pioneers of signal transduction research also identified methylxanthines as competitive inhibitors of PDE activity, and established a potency-ranking order for PDE inhibition by theophylline, theobromine and caffeine (Butcher & Sutherland, 1962). Another early observation was that cyclic AMP could regulate cell activity through its ability to bind to a specific protein kinase (Walsh *et al*., 1968), subsequently termed cyclic AMP-dependent protein kinase (PKA). Although it had already been known for more than 10 years that proteins can be reversibly phosphorylated, this observation by Ed Krebs' group provided a key piece in the jigsaw, allowing a signal transduction pathway linking the first messenger (adrenaline) to second messenger (cyclic AMP), and thence to a cellular response (glycogenolysis) to be elaborated.

Extension of this work subsequently revealed the diversity of receptors linked to changes in cyclic AMP turnover that might be present within a single tissue or cell type. The fact that receptors could either increase or decrease cyclic AMP accumulation within the cell, and that the receptor–AC linkage was not direct, but occurs *via* a family of heterotrimeric guanine nucleotide-binding proteins, termed G-proteins (Rodbell, 1980; see also Milligan & Kostenis, this issue). These findings led from the early concept of the receptor-effector being a single entity to the establishment of the 'universal' tripartite (receptor-G-protein-effector) sequence by which GPCRs were thought to exert the vast majority of their cellular actions (Gilman, 1987).

As with all such evolving areas, development of user-friendly methods was the key to progress and a relatively simple binding assay developed in the U.K. (Brown *et al*., 1971) for cyclic AMP mass, and a method to quantify AC activity (Salomon, 1979) played (and continues to play) an essential role in the investigation of this signalling. One of the first examples of using AC activity as a pharmacological response was an extensive study by Kaumann & Birnbaumer (1974), then at the Department of Physiology, North Western University, Chicago, who characterized the cardiac β-adrenoceptor coupling to this second messenger-generating pathway. Using classical Schild analysis, K_B values for a range of β-adrenoceptor antagonists in the AC assay on cardiac ventricular membranes were shown to closely mirror those obtained using contractility approaches in the intact heart. Although this convinced some of us that the receptor in the AC assay was identical to that driving cardiac contractility, traditional pharmacologists were slow to accept this. Indeed, the suspicion by many of such 'simple assays' quickly extended to ligand-receptor-binding assays when they were extensively developed in the 1970s. British receptor pharmacology in the 1970s and 1980s, in my opinion, remained blunted because of this conservatism, while molecular pharmacology flourished in the U.S.A. and Europe, leading eventually, of course, to the cloning of many GPCRs. Even in today's molecular and now genomic/proteomic era, it has been difficult for the U.K. to genuinely compete in these areas of pharmacology.

The initial picture that emerged for the cyclic AMP pathway was relatively simple, with subsets of GPCRs influencing the activity of AC through either stimulatory G_s or inhibitory G_i proteins. However, the cloning of multiple G-protein α, β and γ subunits, and AC and PDE isoforms, quickly revealed the actual complexity of intracellular signalling cascades. This work also provided the first clear insights into how receptor-mediated changes in intracellular signalling cascades can interact ('crosstalk') on multiple levels. For example, cellular AC activity is regulated not only by G_s/G_i-coupled GPCRs but also by G_q-coupled GPCRs which can affect a subset of isoenzymes of AC through changes in the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) and protein kinase C activity (Cooper *et al*., 1995). Another theme to arise from explorations of cyclic AMP signalling is the fact that the major cyclic AMP target, PKA, can be localized within cells through reversible interactions with anchoring proteins (AKAPs; Wong & Scott, 2004), and effect changes in different cellular compartments, such as the nucleus.

As our present appreciation of cyclic AMP signalling has evolved, a diverse array of potential pharmacological targets has also emerged. Although a variety of experimental agents that are activators or inhibitors of AC, PKA or PKA–AKAP interactions have been developed, selective PDE inhibition has been seen as the most attractive approach giving us drugs such as the PDE5-selective inhibitor sildenafil (see also Boswell-Smith *et al*., this issue).

The enormous importance and impact that cyclic nucleotide and protein phosphorylation research has had is reflected by the award of five separate Nobel Prizes, since 1960: Earl Sutherland (1971) Cyclic AMP; Alfred Gilman & Martin Rodbell (1994) G-Proteins; Edmund Fischer & Edwin Krebs (1992) Kinases and Protein Phosphorylation; Robert Furchgott, Louis Ignarno & Ferid Murad (1998) NO and Cyclic GMP; Arvid Carlsson, Paul Greengard & Eric Kandel (2000) Signal Transduction in the Nervous System.

Phosphoinositide signalling: a multiplicity of second messengers and a British success story!

In the mid-1970s, the British biochemist Michell (1975) published a landmark review linking inositol lipids and cell-surface receptor function. In particular, he argued that inositol lipid hydrolysis was responsible for Ca^{2+} signalling. Michell used pharmacological principles such as specific receptor occupation and response in making this association, and it is

remarkable that, unlike other signalling pathways, phosphoinositide turnover has been hugely influenced by British biomedical scientists, including several located in Pharmacology Departments. Work by a variety of U.K. groups, including those of Rex Dawson (Babraham), Tim Hawthorne (Nottingham) and Bob Michell (Birmingham), provided the biochemical foundations for the discovery of two new second messengers (Irvine, 2003). Evidence for *sn*-1,2-diacylglycerol (DAG) fulfilling a second messenger role arose predominantly from the laboratory of Nishizuka, who discovered that DAG (and phorbol esters) activates a novel protein kinase, named by them as protein kinase C (Nishizuka, 1984). Concurrently, Mike Berridge was spearheading efforts to establish the nature of the water-soluble product of inositol phospholipid turnover, which culminated in the discovery of inositol 1,4,5-trisphosphate (IP_3) as a second messenger (Streb *et al*., 1983).

Subsequently, a family of phospholipase C isoenzymes (β, γ, δ, ε, η and ζ) has been characterized that utilize the minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), to generate IP_3 and DAG. These products fulfil their respective second-messenger roles through IP_3-mediated mobilization of Ca^{2+} from endoplasmic reticular stores and activation by DAG of a subset of isoenzymes of protein kinase C (the conventional and novel subfamilies). Interestingly, PIP_2 has now been shown to regulate, directly, a number of key cell modalities, including the modulation of an array of ion channels (Suh & Hille, 2005). Furthermore, PIP_2 can be 3-phosphorylated by phosphoinositide 3-kinases (PI3Ks) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which, together with its immediate metabolite, phosphatidylinositol 3,4-bisphosphate, has demonstrable second-messenger activities.

The collaboration of Berridge and Irvine (now Pharmacology, Cambridge) with the Schulz lab led to the landmark discovery, of $Ins(1,4,5)P_3$ (Streb *et al*., 1983) as the Ca^{2+}-mobilizing second messenger. This was followed by a decade of discovery, which delineated all of the steps constituting the phosphoinositide cycle and discovered new inositol polyphosphates. The elucidation of the phosphoinositide cycle also revealed a number of novel therapeutic targets, including a potential explanation of the therapeutic action of lithium ions (Li^+) in bipolar disorder (Berridge *et al*., 1989; Nahorski *et al*., 1991). In addition, the inhibition of inositol monophosphatase by Li^+ provided a simple assay for inositol phospholipid hydrolysis by phospholipase C (PLC) activity (Berridge *et al*., 1982). It is remarkable that, unlike the cyclic nucleotides, much of the fundamental work on PLC signalling was completed in U.K. laboratories. The following, very personalized account hopefully gives a flavour of those heady days.

More inositol polyphosphates!

In May 1985, Merck, Sharpe & Dohme opened their Neurosciences Research Centre in Harlow with a symposium on 'Fast and Slow' Cellular Signalling. Geoff Woodruff, Ray Hill and Norman Bowery were among several BPS members recruited by the new Director, Leslie Iversen. It was at this meeting that I met Robin Irvine for the first time, and with Sol Snyder (ringed in photo) (Figure 1) excitedly discussed the potential of the newly discovered PLC signalling pathway.

Figure 1 The Opening of the Merck, Sharpe & Dohme Neurosciences Research Centre, Harlow, U.K. (1985). How many BPS members can you identify?

My lab, at that time, was predominantly using the PLC pathway to characterize GPCR pharmacology, but one of my PhD students (Ian Batty) never accepted the standard method established in the lab to separate inositol phosphates and painstakingly ran out every fraction of extracts from carbachol-stimulated [^{3}H]inositol-labelled rat cerebral cortical slices on Dowex-anion columns. Ian identified a peak that indicated a product, more polar than Ins(1,4,5)P_3, that accumulated very rapidly after stimulation of the muscarinic receptor. Robin Irvine, meanwhile, had been struggling with the identity and source of another 'strange' inositol trisphosphate (Ins(1,3,4)P_3) that accumulated in parotid glands after muscarinic stimulation (Irvine *et al*., 1984). Robin had used a novel HPLC method to separate this isomer from Ins(1,4,5)P_3 and his first reaction to hearing of our results was that Ian had separated the two isomers, but Ian and I were strongly of the view that this was probably a higher polyphosphate, possibly a tetrakisphosphate. After much animated discussion over a few Merck-sponsored beers, we agreed to bring some of our cortex extracts for analysis on Robin's HPLC system at the Babraham Institute. We travelled down in the first week in July 1985 (20 years to the week of writing this article and similar blazing weather) and set the samples running, while we retreated to the village pub! I recall well, despite the beer haze, the 'eureka' moment when we returned and realized that the product only came off the HPLC with a much higher ionic strength buffer (that we had to make up on the spot), and that this must be a tetrakisphosphate (Batty *et al*., 1985). Robin, Ian and I were all seen to be dancing down the corridors of Babraham with excitement, fuelled by the heady mix of IP_4 and strong bitter! Robin went on to identify the mystery peak as Ins(1,3,4,5)P_4, and this neatly suggested a pathway whereby Ins(1,4,5)P_3 was phosphorylated by a 3-kinase activity to Ins(1,3,4,5)P_4, which could then be dephosphorylated to the Ins(1,3,4)P_3 product Robin had previously identified.

Now, 20 years down the line, these new products of Ins(1,4,5)P_3 still seek a convincing functional cellular role (although Robin would certainly challenge that!), but they were the first of a myriad of higher inositol polyphosphates, some of which may be intracellular messengers or perhaps unique contributors to protein phosphorylation (Irvine & Schell, 2001; Saiardi *et al*., 2004). Indeed, the latter is one of Sol Snyder's most recent revelations and reminded me that he too was present at the Merck opening (see Figure 1) and was also strongly linked to this second story of Ins(1,4,5)P_3 signalling.

Receptors and a pharmacology for IP_3

By the mid-1980s it had been accepted that Ins(1,4,5)P_3 is a crucial second messenger that releases Ca^{2+} from stores associated with the endoplasmic reticulum. A number of laboratories including Snyder's and mine reasoned that there should exist specific intracellular receptors for IP_3, and set about developing ligand-binding approaches to identify these receptors. Our approach was again shaped by pharmacological principles and a crucial linkup with Barry Potter, a synthetic medicinal chemist (then in Leicester, and now in Pharmacy/Pharmacology at the University of Bath), allowed a thorough structure-binding analysis to be performed at this intracellular receptor. Not only could we establish the specificity of the binding (high D-/L-Ins(1,4,5)P_3 stereo-specificity and negligible affinity for a large variety of naturally occurring inositol (poly)phosphates), but we could also (together with Ca^{2+} release functional assays – our equivalent of organ bath pharmacological assays) characterize a large number of chemically synthesized inositol polyphosphates (see Nahorski & Potter, 1989; Wilcox *et al*., 1998). Whether such intracellular receptors represent targets for rational drug design still remains an enigma. Despite a good array of full and partial agonists and even $InsP_3$ receptor antagonists, the specificity of such agents and the considerable problem of membrane permeability for synthetic analogues possessing charged phosphate or phosphorothioate moieties still represent a major challenge. However, the pharmacological identity of Ins(1,4,5)P_3-binding sites from cerebellum led to their purification and functional reconstitution by the Snyder lab and more recently the characterization of three distinct IP_3 receptors.

However, the high affinity and specificity of IP_3 receptors for Ins(1,4,5)P_3 has allowed the development of a simple radioreceptor assay (Challiss *et al*., 1988) to quantify IP_3 mass from cell and tissue extracts. The accessibility of mass assays for this messenger as well as its lipid precursor PIP_2 and its kinase-derived product IP_4 has been invaluable in recent investigations of these intracellular pathways and in the evaluation of the enormous number of GPCRs that use this signalling pathway. Whether IP_3 receptor subtype-specific ligands can be developed or whether cell-permeant inhibitors of the enzymes that metabolize IP_3 prove to be useful therapeutic agents (cf. cyclic nucleotide phosphodiesterase isoenzyme/isoform-selective inhibitors) requires a still better understanding of this signalling pathway and its associated proteins. However, perhaps there are clues from the action of Li^+, which features in this third story.

A new lithium?

Another remarkable link to that day in 1985 at Merck, Sharpe & Dohme relates to attempts to develop specific inhibitors of the phosphoinositide signalling pathway. I had also met Ian Ragan, newly recruited by Les Iversen, to head the Biochemistry section. Ian, an enzymologist, was keen to develop inhibitors of the enzyme inositol monophosphatase that was known to be inhibited by Li^+ and was, at that time, proposed to underlie its action in bipolar disorder.

The elucidation of the phosphoinositide cycle, which generates and terminates the actions of IP_3 and DAG, produced an enduring (and still unproven) hypothesis as to how Li^+ exerts an anti-manic action in bipolar disorder. Although lithium salts had been used since the early 1960s to treat manic depression, essentially nothing was known about how Li^+ might exert its therapeutic action in the brain. A series of studies by Bill Sherman culminated in the discovery that Li^+ inhibits inositol monophosphatase (IMPase) activity and that it does so *via* an uncompetitive inhibitory mechanism (Hallcher & Sherman, 1980). In contrast to the much more widely observed competitive mode of inhibition, the action of an uncompetitive inhibitor cannot be overcome by increasing substrate concentration; in fact, as substrate concentration increases the inhibitory grip of an uncompetitive agent is increased (Nahorski *et al*., 1991). Thus, at a therapeutic Li^+ concentration, 0.5–1.0 mM, the inhibition of IMPase

activity should be most marked in subpopulations of neurones exhibiting the highest rates of phosphoinositide turnover (Berridge *et al*., 1989). In the presence of Li^+, two sources of myo-inositol (recycling of the products of PIP_2 hydrolysis, and *de novo* synthesis) are to a greater or lesser extent unavailable and the most active neurones may become wholly reliant on the intracellular pool of myo-inositol and/or its uptake from the extracellular milieu to maintain the inositol phospholipid ($PI/PIP/PIP_2$) pool. A likely consequence of this is that in neurones where the free myo-inositol pool becomes exhausted and myo-inositol transport systems cannot keep pace with receptor-stimulated PLC activity, generation of IP_3 and DAG will be compromised. Indeed, this is exactly what my lab demonstrated in model CHO cells expressing the M_1 muscarinic receptor (Jenkinson *et al*., 1994).

The Merck group (Atack *et al*., 1995) developed a series of IMPase inhibitors to examine whether such agents would mimic the effect of Li^+ on the PI cycle and presumably provide insight into, and possibly intervention of, this action in manic-depressive illness. One such compound (L690,330) and its cell membrane-permeable analogue (L690,488) were shown to be potent competitive inhibitors of IMPase and both resulted in similar agonist-dependent accumulation of $InsP_1$ to Li^+. However, the limitation of blood–brain barrier penetration of such agents *in vivo* precluded their further evaluation and therefore importantly whether inhibition of IMPase *per se* was critical to the therapeutic benefit displayed by Li^+.

So, a causal link between Li^+-induced disruption of neuronal phosphoinositide turnover and mood stabilization in bipolar disorder remains unproven, although some supporting circumstantial evidence has accumulated. However, the change in the response of susceptible subpopulations of neurones to neurotransmitter(s) in the presence of Li^+ may not simply relate to alterations in $IP_3/DAG/Ca^{2+}$ responses *per se*, but through the ability of these intermediates to bring about longer-term neuronal changes through up- and down-regulation at the level of transcription and/or translation. Additionally, the uncompetitive nature of the inhibitory effect may be crucial for Li^+ to exert a targeted effect and may point to a lack of success of the competitive IMPase inhibitors, synthesized as Li^+-mimetics, in treating bipolar disorder, even if pharmacokinetic shortcomings could be overcome.

Intracellular Ca^{2+} homeostasis

Ja Kalzium, das ist alles!

Otto Loewi

Our current appreciation of Ca^{2+} as a ubiquitous intracellular messenger that controls a multitude of activities within cells is commonly traced back to Sidney Ringer, who in 1883 discovered that Ca^{2+} is essential for the maintenance of cardiac contractility *in vitro*. However, it was not until the latter half of the last century that the proteins responsible for regulating $[Ca^{2+}]_i$ and binding Ca^{2+} were identified and characterized (Carafoli, 2002), and a firm understanding of the contributions of extracellular and intracellular organellar Ca^{2+} to Ca^{2+} signalling achieved.

Indeed, despite the universal acceptance following the brilliant work of Ebashi & Endo (1968) that Ca^{2+} was a messenger in skeletal muscle, the notion that this ion might be a universal, intracellular messenger was swept away in the 1960s by the excitement surrounding cyclic AMP. As Michael Berridge (2004) recently wrote, "it was only Howard Rasmussen and Bill Douglas who attempted to stem the cyclic AMP tide". Indeed, it was only a special feature of the 'Fifth International Conference on Cyclic Nucleotides and Protein Phosphorylation', held in Milan in 1983, that first included papers from a (separately funded) symposium on the biochemical and pharmacological control of Ca^{2+} modulation!! Interestingly, it was Howard Rasmussen's sabbatical in the Department of Pharmacology in Cambridge that initiated a collaboration with Michael Berridge to study Ca^{2+} signalling in blowfly salivary glands. Although Mike would be very reluctant to be described as a pharmacologist, his early work used pharmacological principles (and was published in the *British Journal of Pharmacology*) to demonstrate that the gland had two 5-HT receptors utilizing separate second messengers. One used cyclic AMP to drive K^+ transport, whereas the other used Ca^{2+} to open Cl^- channels (Berridge & Heslop, 1981). These blowfly studies were the basis on which the search for a Ca^{2+}-mobilizing second messenger was initiated and assisted by the U.K. network of phosphoinositide signallers, allowed Mike Berridge, Robin Irvine and colleagues to discover $Ins(1,4,5)P_3$ in 1983.

Within 4 years other Ca^{2+}-mobilizing messengers had been proposed, using sea urchin eggs this time! Cyclic adenosine diphosphate ribose (cADP-ribose) and nicotinic acid adenosine dinucleotide phosphate (NAADP) were demonstrated to mobilize Ca^{2+} in these preparations (Clapper *et al*., 1987) and, more recently, work from Antony Galione's group at the Department of Pharmacology, Oxford, has suggested that cholecystokinin signalling in pancreatic acinar cells is initiated by NAADP releasing Ca^{2+} from a lysosome-related organelle and that this Ca^{2+} triggers further Ca^{2+} release from $InsP_3$ and ryanodine receptors (Galione & Ruas, 2005). Again, much of this proposal rests on pharmacological approaches (using specific antagonists of these messengers), but has been recently reinforced by making direct mass assays of these messengers using radioreceptor approaches. Unlike the $InsP_3$ system, our understanding of cADP-ribose and NAADP as messengers in mammalian cells is still incomplete. In particular, even if both messengers are generated by the same enzymatic pathway, the mechanisms of transduction are ill-defined.

A crucial breakthrough in Ca^{2+} signalling was the ability to visualize changes in intracellular Ca^{2+}, first using aequorin isolated from *Aequorea victoria*, and subsequently synthetic fluorescent Ca^{2+} dyes (Cobbold & Rink, 1987). Since the first reported use of aequorin to measure changes in intracellular Ca^{2+} (Ridgway & Ashley, 1967), the rapid advances in this field have provided the reagents necessary to quantify Ca^{2+} changes (Grynkiewicz *et al*., 1985), to observe the spatiotemporal changes in cytoplasmic $[Ca^{2+}]$ that are now believed to allow both amplitude and frequency encoding of signalling information (Berridge *et al*., 2000), and to interrogate Ca^{2+} signatures within specific organelles or microdomains within cells (Pozzan *et al*., 2003). It could be argued that the development and exploitation of these fluorescent and bioluminescent biosensor approaches has done more than any other technology to advance our understanding of cell biology and hence our ability to visualize the cellular consequences of pharmacological manipulation of receptor activity. However,

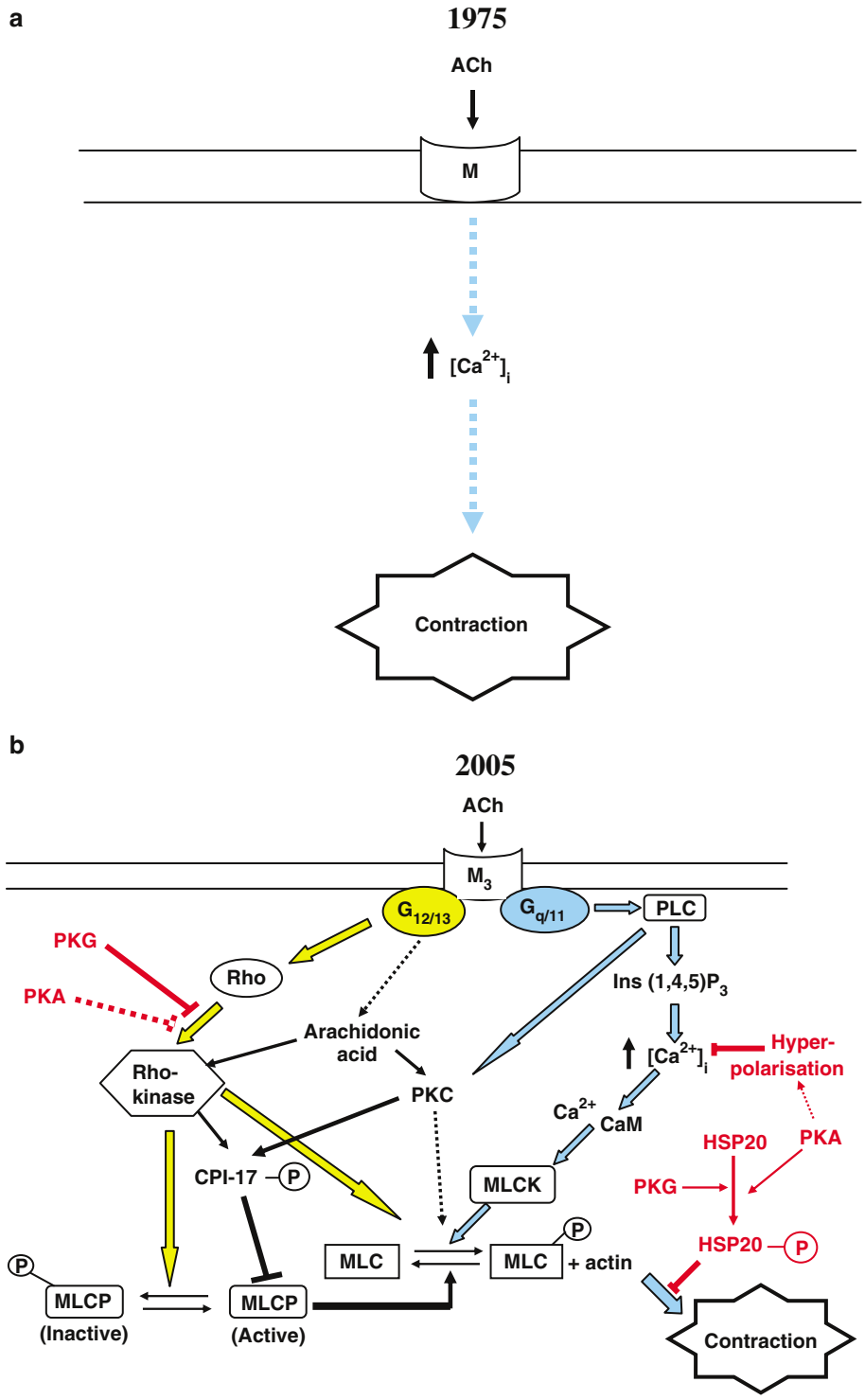

Figure 2 Development of the intracellular signalling mechanisms involved in smooth muscle contraction and relaxation, between 1975 and 2005. In 1975 (a), acetylcholine (ACh) activated a muscarinic receptor (M) in the smooth muscle cell membrane, causing a rise in the intracellular concentration of free calcium and hence contraction of the smooth muscle. After 30 years (b), this simple pathway has at least six identified components (blue arrows) and there are other, separate, pathways (such as those initiated through $G_{12/13,}$ yellow arrows) that influence the final cellular response. Note that PKA and PKG (red arrows) exert inhibition of both the $G_{q/11}$/PLC (blue) pathway and the $G_{12/13}$/Rho (yellow) pathway. M_3, M_3 muscarinic acetylcholine receptor; PLC, phospholipase C; PKC, protein kinase C; PKA, protein kinase A; PKG, protein kinase G; Ins(1,4,5)P_3, inositol (1,4,5) trisphosphate; CaM, calmodulin; CPI-17, smooth muscle-specific phosphopeptide; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; HSP-20, heat shock protein 20. Adapted from Pfitzer (2001).

the real pharmacological successes have been with agents that target specific Ca^{2+} channels (see also Dolphin, this issue).

A 30-year transformation of mechanisms underlying muscarinic receptor-mediated contraction of smooth muscle

Agonist-mediated contraction of smooth muscle has been a central bioassay of the classical pharmacologist for more than 75 years, but it is astonishing how our understanding of the intracellular signalling underlying this fundamental response has been transformed since 1975 (see Figure 2a and b). In that year, when I was appointed to a Lectureship in Leicester, a pharmacologically defined muscarinic receptor was believed to mediate smooth muscle contraction, through a mechanism dependent on extracellular Ca^{2+}. The situation in skeletal muscle was a little more developed, mainly because of the seminal work of Setsuro Ebashi in the 1960s, in which his group was able to show that Ca^{2+} released from the sarcoplasmic reticulum under the influence of action potentials affects troponin and releases the actin filament from its depressed state, resulting in contraction (Ebashi & Endo, 1968). However, in smooth muscle there was little indication of different subtypes of muscarinic receptors, let alone their molecular identity, and the mechanisms of transmembrane signal transduction and intracellular signalling underlying contraction were a void (or a black box) in terms of concept, let alone detail.

By the mid-1980s we knew the identity and probable structure of the M_3 muscarinic, heptahelical receptor that is primarily involved in acetylcholine-mediated contraction. In the same decade, the $G_{q/11}$ protein and the PLC pathway were elucidated and it became clear a little later that the key mechanism of smooth muscle contraction regulation is the phosphorylation/dephosphorylation of Ser-19 of myosin light chain II catalysed by myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). So, as illustrated, in Figure 2b, Ca^{2+} released from intracellular stores by IP_3 binds to calmodulin and activates MLCK. This represents the basic Ca^{2+}-dependent mechanism of contraction, although by the 1990s it became clear that agonists could sensitize contraction at a constant $[Ca^{2+}]_i$ and by 2000 we knew that the same heptahelical receptors could activate another G-protein, $G_{12/13}$, to recruit the monomeric GTPase, Rho, to the plasma membrane. We now know that activation of Rho kinase initiates a still incompletely understood cascade to phosphorylate Thr-697 of the regulatory subunit of MLCP, leading to the latter's inhibition and sensitization to a contractile stimulus.

There are, of course, further intricacies, some of which are still to be elucidated, but it is remarkable to those who have had the privilege to observe and make occasional contributions to this work over the last 20–30 years, to see how the enormous advances in intracellular signalling have transformed our understanding of such physiological systems! But what has this advance in knowledge done for Pharmacology? As is often stated, for instance by Kenakin (1997), ''Pharmacologists should be concerned primarily with the discovery of and quantification of the properties of DRUGS not the physiological systems with which they interact''! My own reply would be, however, to consider the new targets that have arisen from this new understanding of signalling: nitrates that generate NO and increase cyclic GMP to reduce smooth muscle tone via cyclic GMP kinase 1; sidenafil, an inhibitor of cyclic GMP PDE V used widely in erectile dysfunction and inhibitors of Rho-kinase that can reduce blood pressure in hypertensive, but not normotensive, rats.

The future

Over the last few years, it has become clear that the spatial localization of signalling molecules, particularly protein kinases, phosphatases and adaptor molecules, will be key to our understanding of the dynamics of GPCR responses (Wong & Scott, 2004). So, not only the identification of old and new signalling players but also how they are organized in primary cells is currently a particularly active area of research. The approach has changed from the biochemical evaluation of messengers extracted from populations of cells to imaging signalling molecules within single living cells. There are now a host of eGFP-fusion protein translocation or fluorescence resonance transfer (FRET) approaches to investigate the pharmacology of intracellular signalling potentially in microdomains within cells. I believe this cellular approach is the key interface between the molecular identity of signalling proteins and dissecting their role as potential disease targets using genetic knock-out/knock-in approaches in the intact animal.

And what of the new pharmacological agents targeted on signalling pathways? We can already see how this is developing at the level of protein kinases by scanning recent reviews, particularly those published over the last $3\frac{1}{2}$ years in *Nature Reviews/Drug Discovery* (Cohen, 2002; Dancey & Sausville, 2003; Melnikova & Golden, 2004; Mueller *et al*., 2005). At a time when there is a dramatic falloff in the number of new drugs actually entering the market place, will signalling pathways offer the new 'grammar' for drug discovery (Fishman & Porter, 2005)? I do not believe that the next 30 years will see such exciting developments in the identification and understanding of intracellular signalling pathways as the last three decades. However, in 2035 I would anticipate pharmacological targeting of these pathways to be a common therapeutic approach.

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