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MODULATION OF SIGNALLING INITIATED BY PHOSPHOINOSITIDASE-C-LINKED RECEPTORS

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Summary

An extensive group of cell surface receptors are coupled to phosphoinositidase C and thus to the production of the intracellular second messengers inositol 1,4,5-trisphosphate and 1,2-diacylglycerol. While the mechanisms and consequences of phosphoinositidase C activation have been the target of intensive study for over a decade, information is scarce regarding the regulatory processes that modulate this system during receptor stimulation. This situation, however, is now beginning to change. Recent data indicate (a) that Ca^{2+}, mobilized concurrently with activation of phosphoinositidase-C-linked receptors, is a feedback activator and amplifier of phosphoinositide hydrolysis, (b) that rapid desensitization, possibly associated with receptor phosphorylation, regulates phosphoinositidase-C-linked receptors, (c) that receptor internalization can mediate desensitization at later times and (d) that signalling can be regulated at additional sites downstream of phosphoinositidase C. These diverse regulatory events provide the means by which the breakdown of phosphoinositides and cellular responsiveness to their products are controlled during cell stimulation.

Introduction

In recent years, it has emerged that many neurotransmitters, hormones and growth factors communicate with the cell interior *via* receptors coupled to isozymes of phosphoinositidase C (PIC). Activation of these enzymes catalyses phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] hydrolysis and leads to the generation of two intracellular second messengers, inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and 1,2-diacylglycerol (Berridge, 1993; Nishizuka, 1992; Rhee and Choi, 1992; Cockcroft and Thomas, 1992). These are recognised, respectively, by members of the Ins(1,4,5)P_3 receptor and protein kinase C families and, thus, their primary effects are to stimulate Ca^{2+} release from intracellular stores (Berridge, 1993) and to cause protein phosphorylation (Nishizuka, 1992; Hug and Sarre, 1993). In this way, Ins(1,4,5)P_3 and 1,2-diacylglycerol initiate a cascade of intracellular signalling that results in effects as diverse as enhanced secretion and stimulation of cell division.

Mechanistic analyses of the activation of phosphoinositide hydrolysis have been complicated by the fact that there are currently known to be more than ten PIC isozymes

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and three discrete mechanisms by which they can be activated by receptors (Berridge, 1993; Rhee and Choi, 1992; Cockcroft and Thomas, 1992; Wu *et al.* 1993; Carozzi *et al.* 1993). These are (i) activation of PICβ1 by the α-subunits of certain members of the αβγ-heterotrimeric G-protein family, notably G_q and G_{11}, (ii) activation of PICβ2 and PICβ3 by βγ subunits of the same family and (iii) activation of PICγ subtypes by phosphorylation. The first and second mechanisms are characteristically utilized by hormones and neurotransmitters and the third by growth factors (Berridge, 1993).

While these mechanisms have been the target of intensive study for over a decade, surprisingly little unequivocal information has been gathered concerning modulation of signalling during stimulation of PIC-linked receptors. In part this can be accounted for by the complexity of the signalling initiated by phosphoinositide hydrolysis (Nishizuka, 1992) together with the fact that, under certain conditions, PIC may catalyse the hydrolysis of phosphatidylinositol (PtdIns) and phosphatidylinositol 4-phosphate [PtdIns(4)*P*], the precursors of PtdIns(4,5)P_2 (Baird and Nahorski, 1990; Imai and Gershengorn, 1986). Indeed, it has often been assumed that an agonist-occupied receptor is merely a 'switch' that turns PIC 'on' and that no subsequent modulation of PIC activity occurs. As we shall describe, however, this appears not to be the case. It is now becoming clear that, even during the first minute of stimulation, PIC activity is modulated by the increases in cytosolic Ca^{2+} concentration that accompany receptor activation. Conversely, during the same period, desensitization suppresses PIC activity. This rapid desensitization seems to be a feature of all PIC-linked receptors, although its severity varies between receptor types. At later times, receptor internalization and down-regulation modulate PIC activity and signalling is also regulated at points downstream of PIC, notably at the level of the protein kinase C and Ins(1,4,5)P_3 receptor families.

Regulation of PIC activity by cytosolic Ca^{2+}

The general acceptance that phosphoinositide hydrolysis is the cause rather than than an effect of Ca^{2+} mobilization has led to a belief (discussed by Michell *et al.* 1981) that activation of phosphoinositide metabolism is independent of Ca^{2+}. This apparently logical view has, however, been contradicted by a body of data showing that receptor-mediated phosphoinositide hydrolysis, particularly that occurring during prolonged stimulation, is dependent upon the presence of extracellular Ca^{2+} (Meldolesi *et al.* 1991) and can be enhanced by agents that cause an influx of Ca^{2+} into cells (Baird and Nahorski, 1990; Eberhard and Holz, 1988). Indeed, recent studies have shown that submicromolar concentrations of Ca^{2+} can activate purified PIC subtypes reconstituted into phospholipid vesicles (Rhee and Choi, 1992; Martin *et al.* 1991) and can enhance receptor- or G-protein-stimulated phosphoinositide hydrolysis in permeabilized cells (McDonough *et al.* 1988; Fisher *et al.* 1989). Thus, the possibility exists that increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) that occur during the activation of PIC-linked receptors might enhance the already stimulated rate of phosphoinositide hydrolysis.

Agonist-induced increases in $[Ca^{2+}]_i$ are biphasic in many cell types (Merritt and Rink, 1987; Fisher *et al.* 1989; Lambert *et al.* 1990; Stauderman and Pruss, 1990). For example, in SH-SY5Y and SK-N-SH human neuroblastoma cells, which express PIC-linked

muscarinic receptors and which have been used extensively as model systems to study intracellular signalling in neurones (Fisher *et al.* 1989; Lambert *et al.* 1990; Wojcikiewicz *et al.* 1990; Lambert *et al.* 1991), the muscarinic agonist carbachol causes a rapid, but transient, rise in $[Ca^{2+}]_i$ that is maximal at approximately 10s (the 'peak' phase), followed 30–60s later by a lower, but sustained, elevation (the 'plateau' phase). The response of SH-SY5Y cells to carbachol and the sources of Ca^{2+} for this response are shown in Fig. 1A–D. When $[Ca^{2+}]$ in the bathing solution is $1.3 mmol\,l^{-1}$ (Fig. 1A), increases in $[Ca^{2+}]_i$ are biphasic; values of $[Ca^{2+}]_i$ during 'peak' and 'plateau' phases being approximately $500 nmol\,l^{-1}$ and approximately $200 nmol\,l^{-1}$, respectively. Brief pretreatment and co-incubation with the the divalent cation chelator EGTA, which reduces $[Ca^{2+}]$ in the bathing solution to approximately $100 nmol\,l^{-1}$ and which drastically reduces the gradient that drives Ca^{2+} entry, reduces the corresponding values to approximately $200 nmol\,l^{-1}$ and approximately $70 nmol\,l^{-1}$ (Fig. 1B). This obliteration of the 'plateau' phase of Ca^{2+} mobilization by EGTA indicates that it depends upon Ca^{2+} entry; the fact the 'peak' phase is maintained, albeit at a reduced level, indicates that it is in part sustained by release from intracellular Ca^{2+} stores.

Although these responses are typical of those initiated by many types of receptor, the extent of changes in $[Ca^{2+}]_i$ upon stimulation of a particular receptor can vary from cell type to cell type. For example, in Chinese hamster ovary cells transfected with human m3 muscarinic receptor cDNA (CHO-m3 cells), carbachol initiates 'plateau' phases that are very small at a time when Ca^{2+} entry is known to be occurring (Tobin *et al.* 1992). This contrasts with the effects of m3 receptor stimulation in SH-SY5Y cells (Fig. 1) and suggests that additional factors, in particular the rate of Ca^{2+} extrusion from the cell, may control the level of the 'plateau'.

In order to establish directly that intracellular Ca^{2+} stores are involved in the Ca^{2+} responses of SH-SY5Y cells, thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+}-ATPases (Thastrup *et al.* 1990), was used to discharge these stores prior to addition of carbachol (Fig. 1C,D). Within 100s of its addition to SH-SY5Y cells, thapsigargin raises $[Ca^{2+}]_i$ to a new steady state of approximately $175 nmol\,l^{-1}$ and addition of carbachol at this stage causes only a very small and transient increase, indicating that the Ins(1,4,5)P_3-sensitive Ca^{2+} stores normally released during muscarinic receptor activation are almost competely discharged (Fig. 1C). The subsequent slight reduction in $[Ca^{2+}]_i$ to below approximately $175 nmol\,l^{-1}$ (Fig. 1C) is consistent with the idea that muscarinic receptors activate Ca^{2+} pumping from cells (Zhang *et al.* 1992). In cells preincubated with both thapsigargin and EGTA, discharge of intracellular stores does not lead to a persistent increase in $[Ca^{2+}]_i$, presumably because of the loss of the released Ca^{2+} to the extracellular medium and the lack of Ca^{2+} entry (Fig. 1D). Under these conditions, carbachol is totally unable to raise $[Ca^{2+}]_i$, which remains at 50–$70 nmol\,l^{-1}$ throughout the treatment (Fig. 1D).

Clearly, if $[Ca^{2+}]_i$ is involved in regulating PIC activity, then the manipulations illustrated in Fig. 1 should modulate the rate of phosphoinositide hydrolysis during stimulation. Evidence that this is the case is shown in Fig. 2. Carbachol raises the Ins(1,4,5)P_3 concentration in SH-SY5Y cells with biphasic kinetics; the initial 'peak' phase is maximal at approximately 10s and is followed by a sustained 'plateau' phase

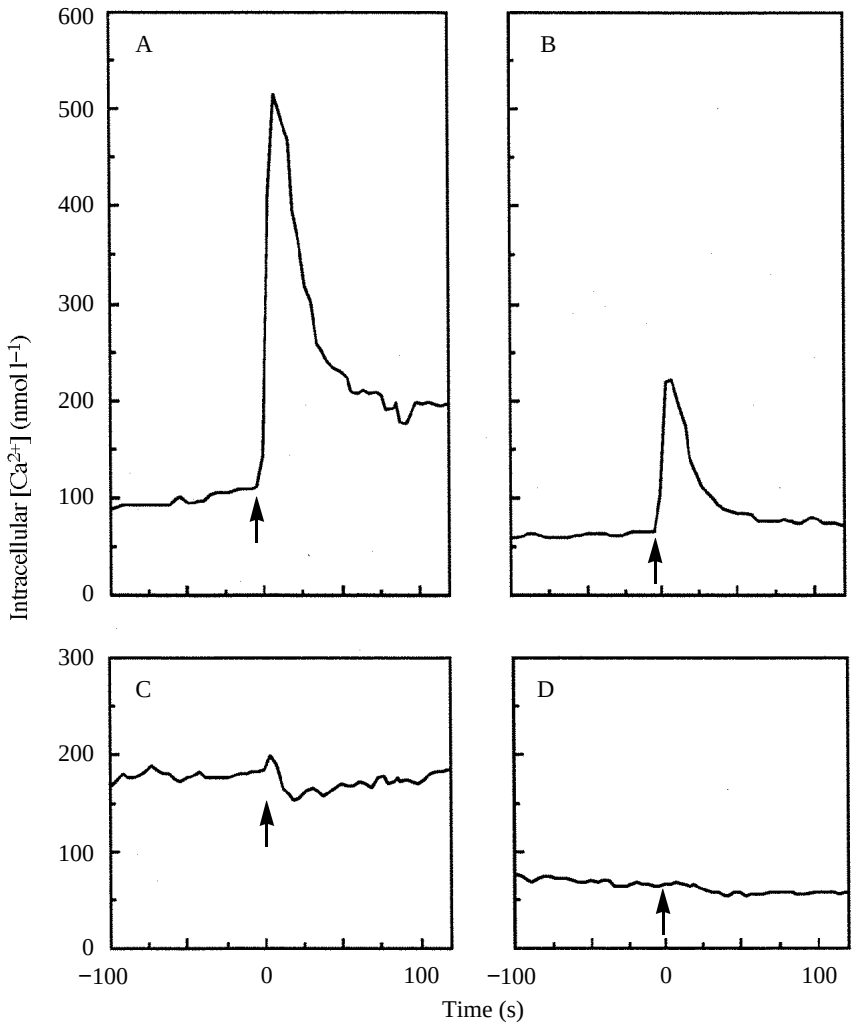

Fig. 1. Kinetics of carbachol-induced Ca^{2+} mobilization in SH-SY5Y cells. $[Ca^{2+}]_i$ in suspensions of SH-SY5Y cells incubated in Krebs–Hepes buffer was measured using the fluorescent Ca^{2+} indicator Fura-2 (Grynkiewicz *et al.* 1985; Lambert *et al.* 1990). As indicated by the arrows at time zero, cells at 37°C were challenged with 1mmol l^{-1} carbachol after no pretreatment (A) or after pretreatment for approximately 4min with 3mmol l^{-1} EGTA (B), 2 μmol l^{-1} thapsigargin (C) or 3mmol l^{-1} EGTA plus 2 μmol l^{-1} thapsigargin (D). Addition of 3mmol l^{-1} EGTA to Krebs–Hepes buffer reduced $[Ca^{2+}]$ to 101±9nmol l^{-1} (N=5). Data shown are representative of three independent experiments that yielded qualitatively identical results.

from 60 to 300s at a level 30–50% of the maximum (Fig. 2A). Pretreatment with EGTA does not alter the 'peak' phase but reduces the 'plateau' phase by approximately 70 % (Fig. 2B). Similar effects can be seen after pretreatment and co-incubation with 2.5mmol l^{-1} Ni^{2+}, which blocks the channels that mediate agonist-induced Ca^{2+} entry (Lambert *et al.* 1990). These data establish that Ca^{2+} entry can support the 'plateau' phase

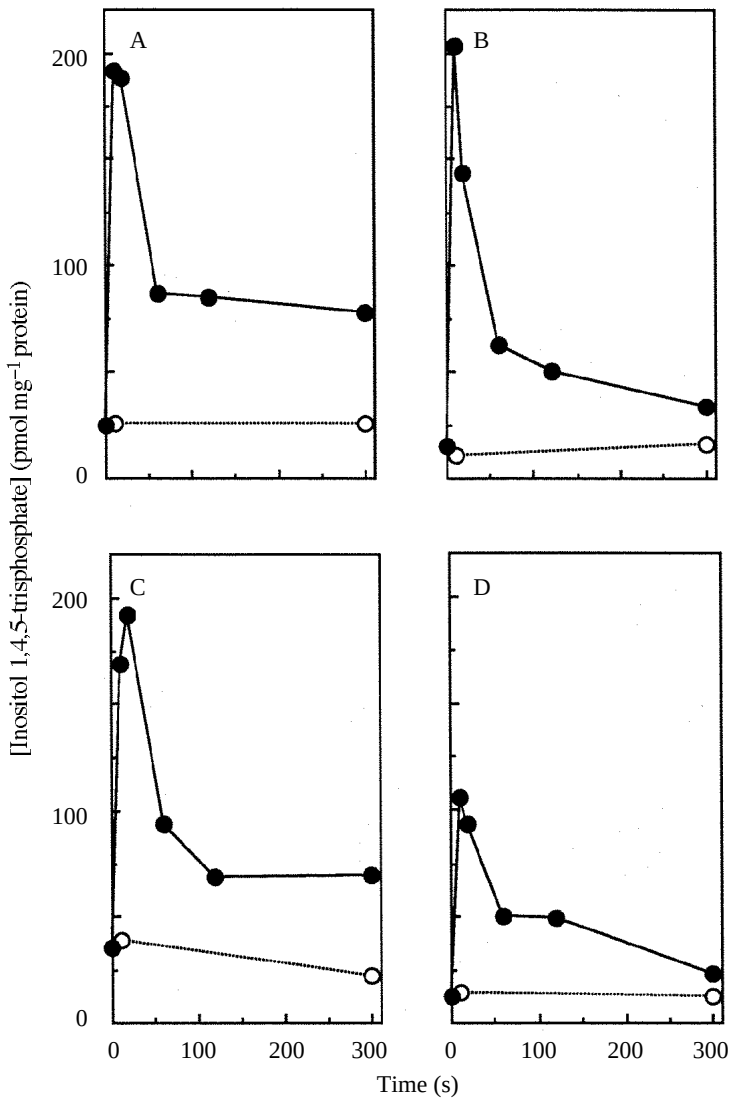

Fig. 2. Kinetics of carbachol-induced increases in Ins(1,4,5)P_3 mass in SH-SY5Y cells. Suspensions of cells in Krebs–Hepes buffer were preincubated for 4min at 37˚C with vehicle (A), 3mmol l^{-1} EGTA (B), 2 μmol l^{-1} thapsigargin (C) or EGTA plus thapsigargin (D). Samples were then incubated at 37˚C without (○) or with 1mmol l^{-1} carbachol (●). Incubations were then terminated and Ins(1,4,5)P_3 mass was assessed (Challiss *et al*. 1990). Data shown are the means of duplicate incubations from an experiment representative of three that yielded similar results. EGTA reduced Ins(1,4,5)P_3 concentration during the 'plateau' phase (at 300s) by 67±6% and EGTA plus thapsigargin reduced Ins(1,4,5)P_3 concentration during the 'peak' phase (at 10s) by 47±4% (N=3).

of phosphoinositide hydrolysis. While pretreatment with thapsigargin does not alter the Ins(1,4,5)P_3 response to carbachol (Fig. 2C), co-incubation with thapsigargin plus EGTA reduces the 'peak' phase of the Ins(1,4,5)P_3 response by approximately 50% (Fig. 2D).

Thus, Ca^{2+} from intracellular stores may also be involved in enhancing Ins(1,4,5)P_3 formation during receptor activation.

In order to confirm that Ins(1,4,5)P_3 formation is sufficiently sensitive to $[Ca^{2+}]$ to be regulated by the effects of inhibition of store release and entry, the Ca^{2+} sensitivity of PIC must be examined directly. This has been done in a number of cell types following permeabilization (McDonough *et al.* 1988; Fisher *et al.* 1989). In electrically permeabilized SH-SY5Y cells (Wojcikiewicz *et al.* 1990), carbachol-stimulated Ins(1,4,5)P_3 formation is very sensitive to changes in $[Ca^{2+}]$ (EC_{50}=36±3nmol l^{-1}; N=5). On this basis, inhibition of Ca^{2+} entry into SH-SY5Y cells during stimulation with carbachol would suppress the rate of Ins(1,4,5)P_3 formation, but only during the 'plateau' phase; EGTA (Fig. 1B) reduces 'plateau' $[Ca^{2+}]_i$ in intact cells from 150–200nmol l^{-1} to 50–70nmol l^{-1} and this correlates with a 30–40% reduction in carbachol-stimulated Ins(1,4,5)P_3 formation in permeabilized cells. In contrast, 'peak' $[Ca^{2+}]_i$ (500–600nmol l^{-1}), while being reduced by inclusion of EGTA (Fig. 1B), remains at or above 200nmol l^{-1} and Ins(1,4,5)P_3 formation is insensitive to changes in $[Ca^{2+}]$ in this range. Only when the 'peak' of the Ca^{2+} response is totally blocked and $[Ca^{2+}]_i$ is maintained at approximately 60nmol l^{-1} with thapsigargin and EGTA (Fig. 1D) would, on the basis of the data from permeabilized cells, the 'peak' phase of the Ins(1,4,5)P_3 response be suppressed.

Overall, these data indicate that in SH-SY5Y cells muscarinic-receptor-stimulated PIC activity is sensitive enough to $[Ca^{2+}]$ for concurrently mobilized Ca^{2+} to enhance the rate of PtdIns(4,5)P_2 hydrolysis. The same conclusion can be drawn from studies with other systems, for example SK-N-SH cells (Fisher *et al.* 1989) and chick heart cells (McDonough *et al.* 1988). It must be stressed, however, that biphasic Ins(1,4,5)P_3 formation is *not* a consequence of biphasic changes in $[Ca^{2+}]_i$. This can be concluded from the experiments with thapsigargin-pretreated SH-SY5Y cells in which carbachol-induced increases in $[Ca^{2+}]_i$ were essentially blocked while the Ins(1,4,5)P_3 response remained biphasic. These results are very similar to those in gastrin-stimulated rabbit parietal cells in which thapsigargin had no effect on the profile of Ins(1,4,5)P_3 formation (Roche *et al.* 1993). Thus, Ins(1,4,5)P_3 formation appears to be intrinsically biphasic; Ca^{2+} derived from intracellular or extracellular sources merely amplifies the response.

Initially, enhancement of phosphoinositide hydrolysis by increases in $[Ca^{2+}]_i$ seems paradoxical as Ins(1,4,5)P_3 releases intracellular Ca^{2+} stores (Berridge, 1993) and is involved in gating Ca^{2+} entry, either indirectly following conversion to inositol 1,3,4,5-tetrakisphosphate (Luckhoff and Clapham, 1992) or *via* capacitative refilling (Hoth and Penner, 1992), or perhaps directly *via* Ins(1,4,5)P_3-gated plasma membrane Ca^{2+} channels (Khan *et al.* 1992). Thus, in the continuous presence of agonist and with normal extracellular $[Ca^{2+}]$, this process might be expected to lead to uncontrolled feedback activation of PIC. Clearly, however, this does not occur, suggesting that the degree to which PtdIns(4,5)P_2 is hydrolysed is in some way limited. It may be that the mechanism providing this constraint also accounts for the fact that, in SH-SY5Y and other cells, agonist-induced changes in Ins(1,4,5)P_3 concentration are intrinsically biphasic (Fu *et al.* 1988; Donie and Reiser, 1991; Menniti *et al.* 1991; Lambert *et al.* 1991). Such a mechanism may also account for the fact that upon stimulation of some receptors changes

in Ins(1,4,5)P_3 concentration are monophasic, merely consisting of a 'peak' followed by a return within 60s to basal levels (Sugiya *et al.* 1987; Menniti *et al.* 1991). For these receptors, PtdIns(4,5)P_2 hydrolysis appears to be rapidly and completely terminated. Taken together, these data suggest that rapid desensitization may constrain the rate of phosphoinositide hydrolysis and that the degree to which desensitization occurs varies from receptor to receptor.

Desensitization of phosphoinositide responses

Rapid desensitization

Based on the precedent set by other G-protein-linked receptors, notably the well-characterised β-adrenoceptor which stimulates adenylate cyclase and which desensitizes during the first few minutes of activation (Kassis and Fishman, 1984; Hausdorff *et al.* 1990), one might expect that similarly rapid events would regulate PIC-linked receptors. This has certainly been borne out in the action of some receptors, for example that for substance P in rat pancreatoma AR4-2J cells, which mediate responses that virtually completely disappear within 1min (Menniti *et al.* 1991). In contrast, stimulation *via* other receptors (for example, muscarinic, cholecystokinin and bombesin) is sustained (Menniti *et al.* 1991), albeit with the characteristic 'peak' and 'plateau' phases seen in SH-SY5Y cells (Fig. 2). Similarly, in bovine adrenal chromaffin cells, responses to angiotensin II are rapidly and almost completely suppressed, whereas responses to histamine are more robust and persistent (Stauderman and Pruss, 1990). These data illustrate two points: first, that apparent desensitization is rapid and, second, that receptor function can be suppressed either completely or only partially. Direct evidence that monophasic and biphasic Ins(1,4,5)P_3 formation results from rapid desensitization comes from recent studies showing that brief pretreatment of cells either obliterates or reduces, respectively, subsequent responses to the same agonist (Donie and Reiser, 1991; Menniti *et al.* 1991; Tobin *et al.* 1992). From such studies it can also be concluded that desensitization of PIC-linked receptors is 'homologous' rather than 'heterologous', since in almost all cases pretreatment of cells with a particular agonist suppresses subsequent responses to that agonist only (Sugiya *et al.* 1987; Dillon-Carter and Chuang, 1989; Menniti *et al.* 1991).

A major advance in the analysis of rapid desensitization has been made recently by directly measuring total phosphoinositide hydrolysis and thus PIC activity over the first minute of agonist stimulation. This has been acheived by using an assay (total inositol phosphate accumulation in the presence of Li^+) that has been employed for over a decade (Nahorski *et al.* 1991), but with the difference that the initial rate of accumulation is now being assessed. By virtue of its ability to inhibit inositol monophosphatase uncompetitively, Li^+ blocks the final step in the recycling of inositol and causes the build-up of inositol phosphates (Nahorski *et al.* 1991). Thus, total inositol phosphate accumulation in the presence of Li^+ ($[\text{inositol phosphate}]_{\text{total}}$) provides a measure of the rate of total phosphoinositide breakdown and, by inference, PIC activity. Indeed, as PtdIns(4,5)P_2 breakdown accounts for the vast majority of the phosphoinositides hydrolysed in the first few minutes following receptor activation (Imai and Gershengorn, 1986; Hughes and Putney, 1989; Batty and Nahorski, 1989), $[\text{inositol phosphate}]_{\text{total}}$ at

early times approximates to the rate of PtdIns(4,5)P_2 hydrolysis. This popular method of assessing phosphoinositide hydrolysis is, however, not without its problems. In particular, perturbation of inositol recycling by Li^+ can inhibit phosphoinositide synthesis and can lead to an underestimation of receptor-mediated effects (Nahorski *et al.* 1991). However, acute measurement of $[\text{inositol phosphate}]_{\text{total}}$ does provide a valid measure of the rate of phosphoinositide hydrolysis, as the compromising effects of Li^+ will be minimal at early times.

Using $[\text{inositol phosphate}]_{\text{total}}$ as a measure of PIC activity in AR4-2J cells exposed to bombesin (Menniti *et al.* 1991), it has been shown that the initial rate of phosphoinositide hydrolysis is approximately six times greater than that observed after 20s, suggesting that the bombesin receptor rapidly, but partially, desensitizes. Similarly, in SH-SY5Y cells, $[\text{inositol phosphate}]_{\text{total}}$ in response to carbachol is biphasic (Fig. 3), the initial rate (from 0 to 20s) being approximately five times greater than that of the secondary rate (from 30 to 300s).

While Fig. 3 shows that phosphoinositide hydrolysis is biphasic and indicates that the PIC-linked muscarinic receptors expressed in these cells rapidly but partially desensitize, the data also suggest that this leads directly to the complex changes in Ins(1,4,5)P_3

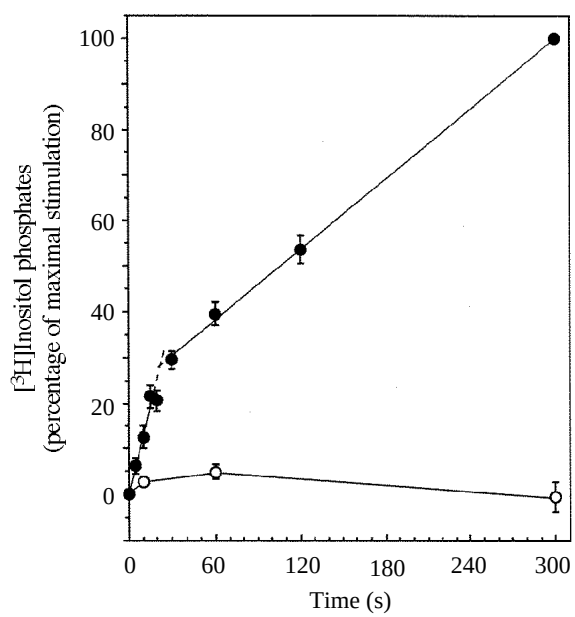

Fig. 3. Kinetics of carbachol-induced total [^{3}H]inositol phosphate accumulation in SH-SY5Y cells. Suspensions of [^{3}H]*myo*-inositol-labelled SH-SY5Y cells in Krebs–Hepes buffer (Wojcikiewicz *et al.* 1990) were preincubated for 3min at 37°C with 10mmol l^{-1} Li^+. Samples of cells were then incubated without (○) or with (●) 1mmol l^{-1} carbachol at 37°C for 5–300s. Incubations were terminated and the accumulation of total [^{3}H]inositol phosphates was assessed (Batty and Nahorski, 1989). Data shown are mean ± S.E.M. of three independent experiments. Basal (*t* 0 s) and stimulated (*t* 300s) total [^{3}H]inositol phosphate accumulation were 4800±500 and 18200±2900disintsmin^{-1}, respectively (*N*=3).

concentration during stimulation. This can be concluded because (i) at early times PtdIns(4,5)P_2 is the primary substrate for PIC and (ii) direct evidence that Ins(1,4,5)P_3 metabolism is modulated in intact cells during activation of PIC-linked receptors is lacking (Shears, 1992). Further, the transition between the two rates of inositol phosphate production (at approximately 20s) correlates temporally with biphasic changes in Ins(1,4,5)P_3 concentration (Fig. 2A), Ins(1,4,5)P_3 mass peaking at 10s and then falling to a lower but maintained level within 1min. Clearly, in the face of a constant capacity to metabolize Ins(1,4,5)P_3, biphasic PtdIns(4,5)P_2 hydrolysis would cause the concentration of this second messenger to peak and then to decline to a lower plateau.

Mechanisms of rapid desensitization

There are a number of plausible mechanisms by which the properties of receptor–PIC coupling could be modulated to account for the differences in responsiveness to agonists during 'peak' and 'plateau' phases.

For example, PIC-β_1 is now known to stimulate the GTPase activity of G_q and G_{11} and it has been suggested that PtdIns(4,5)P_2 may inhibit this activity of PIC (Berstein *et al.* 1992). Thus, the 30–50% reductions in PtdIns(4,5)P_2 concentration that occur in the seconds following receptor activation (Michell *et al.* 1981; Martin, 1983; Chilvers *et al.* 1991) may alter the efficiency of signal transduction.

The most attractive candidate, however, for the modulatory mechanism is rapid receptor phosphorylation, since this follows the precedent set by the comprehensively studied β-adrenoceptor/adenylate cyclase system (Hausdorff *et al.* 1990), where occupation of a receptor with agonist induces rapid β-adrenoceptor phosphorylation at multiple sites, this effect being catalysed by two kinases – protein kinase A and β-adrenoceptor kinase (βARK). As a result of these discrete phosphorylation events, which are half-maximal at approximately 2min and less than 20s, respectively (Roth *et al.* 1991), the receptor is uncoupled from its cognate G-protein and, over a period of minutes, adenylate cyclase activation is diminished (Kassis and Fishman, 1984).

It has been demonstrated recently that certain PIC-linked receptors are phosphorylated; notably, cholecystokinin receptors in intact rat pancreatic acinar cells (Klueppelberg *et al.* 1991), m3 muscarinic receptors in intact CHO-m3 cells (Tobin and Nahorski, 1993) and purified substance P receptors reconstituted with βARK (Kwatra *et al.* 1993). While the latter study by no means proves that βARK phosphorylates PIC-linked receptors under physiological conditions, it is entirely consistent with the studies on CHO-m3 cells, in which the m3 receptor is phosphorylated in an agonist-dependent manner by a kinase distinct from protein kinases A, protein kinase C or other Ca^{2+}-dependent kinases (Tobin and Nahorski, 1993).

Perhaps the most significant finding, however, is that phosphorylation of m3 receptors in intact cells can occur within seconds of agonist application and is, therefore, fast enough to account for rapid desensitization of the sort illustrated in Fig. 3. The cholecystokinin receptor behaves similarly, in that it is rapidly phosphorylated (Klueppelberg *et al.* 1991) and initiates biphasic phosphoinositide responses (Menniti *et al.* 1991). Thus, receptor phosphorylation may be the mediator of this regulatory event, perhaps by causing partial uncoupling of the m3 receptor from G_q/G_{11}. Indeed, it is

possible that the degree to which receptors are uncoupled from G_q/G_{11} following phosphorylation varies from receptor to receptor. This could explain why some receptors (for example, that for substance P) become almost completely desensitized within the first minute of agonist stimulation (Sugiya *et al.* 1987).

Finally, the linkage of PIC activity to cytosolic Ca^{2+} concentration may influence rapid desensitization. Clearly, if different receptors initiate Ca^{2+} responses that differ quantitatively and/or spatially, then variation in the severity of rapid desensitization might be expected. This may account for the finding in adrenal chromaffin cells that the effects of angiotensin II on phosphoinositide hydrolysis are desensitized to a much greater extent than those of histamine (Stauderman and Pruss, 1990). In these cells, histamine promotes prolonged and global increases in cytosolic Ca^{2+} concentration, whereas the effects of angiotensin II are more transient and polarized (Stauderman and Pruss, 1990; Cheek, 1991). It appears, then, that during the period over which rapid desensitization occurs, histamine receptors more effectively stimulate Ca^{2+} entry than do angiotensin II receptors. Thus, for histamine receptors, sustained increases in cytosolic $[Ca^{2+}]$ will oppose suppressive effects on the rate of PtdIns(4,5)P_2 hydrolysis and cause responses to desensitize only partially. It remains to be determined whether the same argument can be applied to other cell types where similar variation in the severity of desensitization has been noted.

Receptor internalization and down-regulation

A reduction in the number of PIC-linked receptors at the cell surface is an additional way in which desensitization can occur. For example, transfer of α_1-adrenoceptors (Leeb-Lundberg *et al.* 1987) and muscarinic receptors (Eva *et al.* 1990) to a vesicular site in the cell interior (internalization) occurs with a half-time of approximately 10min in smooth muscle cells and cultured neurones, respectively, and correlates temporally with reductions in phosphoinositide responses. However, internalization is clearly not the reason for rapid partial densensitization, since in CHO-m3 cells, in which brief pretreatment with carbachol partially suppresses muscarinic receptor responsiveness, m3 receptor numbers at the cell surface remain unchanged for the first 10min of stimulation (Tobin *et al.* 1992).

As with receptors linked to adenylate cyclase (Hausdorff *et al.* 1990), prolonged stimulation can lead to a portion of internalized PIC-linked receptors being lost from the cell. This down-regulation typically occurs after stimulation for hour-long periods and, interestingly, has been shown to occur in parallel with changes in the abundance of $G_q\alpha$ and $G_{11}\alpha$ (Mullaney *et al.* 1993).

Although the molecular events that initiate internalization have yet to be defined, a recent analysis of human m1 muscarinic receptors has revealed that deletion of nine amino acids from a region known to be involved in coupling to G_q/G_{11} is sufficient to severely impair internalization without affecting agonist binding affinity or $[\text{inositol phosphate}]_{total}$ (Lameh *et al.* 1992). Interestingly, this region is rich in serine and threonine, raising the possibility that one or more of these amino acids might be phosphorylated. Thus, as well as being associated with rapid desensitization, phosphorylation may initiate receptor internalization.

Modulation of signalling downstream of PIC

In spite of receptor desensitization, internalization and down-regulation, agonist-stimulated PtdIns(4,5)P_2 hydrolysis can be maintained for hour-long periods, as indicated by a sustained increase in Ins(1,4,5)P_3 concentration mediated by those receptors that only partially desensitize (Wojcikiewicz and Nahorski, 1991; Tobin *et al.* 1992). Levels of 1,2-diacylglycerol can similarly be elevated persistently, albeit from sources not limited to PtdIns(4,5)P_2 (Kiley *et al.* 1991; Nishizuka, 1992). This raises the possibility that the actions of the intracellular proteins that recognise Ins(1,4,5)P_3 and 1,2-diacylglycerol might also be modulated by chronic activation of PIC-linked receptors.

For the protein kinase C family, it has been known for some time that chronic pretreatment of cells with phorbol esters causes a decrease in the cellular concentration of protein kinase C isozymes (Young *et al.* 1987; Hug and Sarre, 1993). These powerful activators, which mimic 1,2-diacylglycerol, appear to cause down-regulation of most if not all protein kinase C isozymes, albeit to differing extents and with different time courses (Huang *et al.* 1989; Akita *et al.* 1990). The mechanistic basis of this down-regulation has been examined in detail and seems to reflect accelerated degradation of active (membrane-bound) protein kinase C by the Ca^{2+}-activated neutral protease calpain (Croall and Demartino, 1991). As phorbol esters leave both protein kinase C mRNA levels and the rate of protein kinase C synthesis unchanged (Young *et al.* 1987), increased proteolysis causes its depletion.

Less is known regarding the regulation that might occur during activation of PIC-linked receptors. However, chronic stimulation of thyrotropin-releasing hormone receptors in GH_4C_1 rat pituitary cells has been reported to cause protein kinase C down-regulation and, intriguingly, this is highly specific to a particular subtype; only the ϵ-isozyme is down-regulated; levels of the α-, β- and δ-isozymes remain unchanged (Akita *et al.* 1990; Kiley *et al.* 1991). It is perhaps significant that 1,2-diacylglycerol concentration remains elevated for up to 12h in the presence of thyrotropin-releasing hormone, perhaps providing the means by which ϵ-protein kinase C is persistently activated and proteolysed (Kiley *et al.* 1991). Although ϵ-protein kinase C down-regulation appears to be linked with reduced transcription of certain genes (Kiley *et al.* 1991), it is not yet known whether such a specific change can sensitize cells to the effects of PIC-linked receptors and reduce the rate at which they are desensitized, as is the case after long-term exposure of certain cells to phorbol esters (Hepler *et al.* 1988).

Evidence in support of Ins(1,4,5)P_3 receptor desensitization has come from experiments showing that the Ca^{2+}-releasing action of Ins(1,4,5)P_3 is suppressed following chronic muscarinic stimulation of rabbit pancreatic acinar and SH-SY5Y cells (Willems *et al.* 1989; Wojcikiewicz and Nahorski, 1991). In SH-SY5Y cells, carbachol-pretreatment halves maximal Ins(1,4,5)P_3-induced Ca^{2+} mobilization and decreases the potency of Ins(1,4,5)P_3 with half-maximal and maximal effects at approximately 3h and 6 h, respectively (Wojcikiewicz and Nahorski, 1991). This suppression of Ca^{2+} release also reflects down-regulation, since it correlates temporally with reductions in the number of high-affinity Ins(1,4,5)P_3 binding sites (Wojcikiewicz and Nahorski, 1991) and type I Ins(1,4,5)P_3 receptor immunoreactivity (Wojcikiewicz *et al.* 1992). It remains to be

determined whether chronic PIC activation reduces the rate of type I Ins(1,4,5)P_3 receptor synthesis or whether, like ϵ-protein kinase C, it is degraded more rapidly. In this regard, it is significant that the Ins(1,4,5)P_3 receptor has recently been shown to be cleaved by calpain (Magnusson *et al.* 1993). Development of antibodies against Ins(1,4,5)P_3 receptor subtypes II (Sudhof *et al.* 1991) and III (Blondel *et al.* 1993) will enable the specificity of this regulation to be determined.

Whether Ins(1,4,5)P_3 receptor down-regulation is occurs in other cell types is not yet known. However, it seems likely that such regulation will be associated with those receptors (e.g. muscarinic and bombesin) that are only partially desensitized during chronic stimulation. In cells with these receptors, in which agonists can persistently elevate Ins(1,4,5)P_3 concentration, decreasing responsiveness to Ins(1,4,5)P_3 represents a potentially very important mechanism for limiting Ca^{2+} release from internal stores and, thus, for limiting the role of Ca^{2+} as an intracellular signal.

Conclusions

Diverse mechanisms modulate the signalling initiated by PIC-linked receptors. Some of these mechanisms, for example the receptor internalization and eventual down-regulation that occur during long-term treatment with agonists, are paralleled by receptors linked to other effectors – notably, adenylate cyclase. In contrast, other modulatory features seem to be unique to PIC-mediated signalling. In particular, two opposing factors appear to regulate phosphoinositide hydrolysis during the first minute of muscarinic receptor stimulation. The first is an increase in $[Ca^{2+}]_i$ that enhances PIC-catalysed PtdIns(4,5)P_2 hydrolysis. The second is a decrease in the stimulated rate of phosphoinositide hydrolysis that occurs after approximately 20s and which seems to vary from receptor to receptor in its severity. Finally, for those receptors that only partially desensitize, down-regulation of Ins(1,4,5)P_3 receptor and protein kinase C subtypes provides a means by which both Ca^{2+} release from internal stores and the roles of Ca^{2+} and 1,2-diacylglycerol as intracellular signals can be limited.

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