Different receptors use inositol trisphosphate to mobilize Ca^{2+} from different intracellular pools

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In cells expressing different receptors linked to Ins(1,4,5)P_3 formation, maximal stimulation of any one of them often releases all the Ins(1,4,5)P_3-sensitive Ca^{2+} stores, suggesting that Ins(1,4,5)P_3 is used similarly by many receptors. In single HEK-293 cells, ATP and carbamylcholine (CCh) stimulated Ca^{2+} release from intracellular stores via a pathway that was entirely dependent on Ins(1,4,5)P_3. After stimulation with maximal concentrations of ATP or CCh in Ca^{2+}-free medium, there was no response to a second stimulation with the same agonist, indicating that each agonist had emptied the Ins(1,4,5)P_3-sensitive stores to which it had access. However, the Ca^{2+} release evoked by the second agonist was unaffected by prior stimulation with the first. We conclude that Ins(1,4,5)P_3 mediates the effects of both receptors, but Ins(1,4,5)P_3 is more versatile than hitherto supposed, because the spatial organization of the signalling pathways apparently allows Ins(1,4,5)P_3 made in response to each agonist to interact with different Ins(1,4,5)P_3 receptors.

Key words: ATP, Ca^{2+} stores, HEK-293 cell, muscarinic receptor, spatial organization.

INTRODUCTION

The second-messenger concept, first enunciated by Sutherland after his discovery that cAMP linked receptors to stimulation of glycogen breakdown in liver [1], has underpinned analyses of signalling pathways for more than 20 years. A key feature of the concept as it has been extended to other second messengers is that a huge number of different receptors regulate cellular activity by causing changes in the intracellular concentration of rather few intracellular signalling molecules [2]. Second messengers are, in effect, a common intracellular currency used by many different receptors in many cells to control almost every aspect of cellular behaviour. It follows from these original ideas that, within a single cell, the same second messenger made in response to activation of one class of receptors would be expected to have the same functional effect as that made in response to activation of another class of receptors. There is, however, now abundant evidence that intracellular messengers are not uniformly distributed within cells and that the resulting spatial organization of these signals is profoundly important. Cytosolic Ca^{2+} signalling, for example, can involve very private exchanges of Ca^{2+} between Ca^{2+} channels in the plasma membrane [3] or endoplasmic reticulum [4] and other organelles. Multivalent anchoring proteins are widely used to direct signalling proteins to specific intracellular locations, allowing spatially restricted decoding of second messengers [5]. A large family of A-kinase-anchoring proteins (AKAPs) ensure precise targetting of cAMP-dependent protein kinase [6], for example, and proteins containing PDZ domains are widely used to assemble complexes of signalling proteins [7]. In short, compartmentalization of intracellular signalling proteins allows second messengers to act locally within cells and thereby endows them with far greater versatility than first envisaged.

Ins(1,4,5)P_3 is the second messenger that is most often responsible for linking the receptors in the plasma membrane that stimulate phosphoinositide hydrolysis with the release of Ca^{2+} from intracellular stores [8]. The increases in cytosolic Ca^{2+} concentration that follow binding of Ins(1,4,5)P_3 to its own intracellular receptors are often complex, because Ca^{2+} itself both stimulates and inhibits Ins(1,4,5)P_3 receptors [9]. Nevertheless, despite some evidence that different receptors may trigger different patterns of Ca^{2+} release within single cells [10], it has been generally assumed that Ins(1,4,5)P_3 evokes the same intracellular response whichever receptor initiates its formation. The present results suggest that Ins(1,4,5)P_3 made in response to activation of different receptors stimulates Ca^{2+} release from different intracellular Ca^{2+} pools.

EXPERIMENTAL

HEK-293 cells (European Collection of Animal Cell Cultures) were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 nutrient mix ('DHM'; Gibco BRL) supplemented with 10% (v/v) foetal-calf serum and glutamine (2 mM). For measurement of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), cells were plated (2.5×10^6 cells/ml) on to glass coverslips coated with poly-L-lysine, and, when the cells were confluent (2–3 days), they were loaded with fura 2 by incubation at 20 °C for 1 h with fura 2 acetoxymethyl ester (2 μM) dissolved in extracellular medium (ECM: 130 mM NaCl/5.4 mM KCl/0.8 mM NaH_2PO_4/1.8 mM $CaCl_2$/0.9 mM $MgSO_4$/10 mM glucose/20 mM Hepes, pH 7.4). The cells were then washed and, after 30 min in ECM, they were used for experiments. Single-cell fluorescence imaging at 20 °C was performed using a MetaFluor system (Universal Imaging Corp., West Chester, PA, U.S.A.). Corrections for background fluorescence and calibration of fluorescence ratios ($R_{340/380}$) to $[Ca^{2+}]_i$ were performed as previously described [11]. In order to correct for variability between individual cells, all responses are expressed relative to the increase in $[Ca^{2+}]_i$ evoked in the same cell by a maximal concentration of carbamylcholine (CCh) in ECM, as described previously [11]. The effects of stimuli on [^{3}H]Ins(1,4,5)P_3 formation were determined as previously described [11].

Abbreviations used: CCh, carbamylcholine; ECM, extracellular medium; AKAPs, A-kinase-anchoring proteins; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; NAADP, nicotinic acid–adenine dinucleotide phosphate.

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RESULTS AND DISCUSSION

Both ATP via endogenous purinoceptors and CCh via the endogenous M_3 muscarinic receptors of HEK-293 cells [11a], evoked an increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) that persisted in the absence of extracellular Ca^{2+} (Figure 1A). Both agonists, in common with many others [8], therefore stimulate release of Ca^{2+} from intracellular stores. Whereas almost all cells ($96 \pm 5\%$) responded to CCh, fewer ($71 \pm 7\%$) responded to ATP, although with some cell passages (Figure 1C) the fraction of cells that responded to ATP increased to up to 93%. Both ATP and CCh stimulated Ins(1,4,5)P_3 formation; treatment for 15 s with maximally effective concentrations of ATP (10 μM) or CCh (100 μM) caused the [^{3}H]Ins(1,4,5)P_3 levels of [^{3}H]Ins-labelled cells to increase to $159 \pm 23\%$ and $250 \pm 31\%$ of the basal level respectively ($n = 6$). The Ca^{2+} mobilization evoked by either ATP or CCh was blocked by U73122 (3 μM), an inhibitor of phospholipase C [12] (Figures 1B and 1C). We conclude that the ability of both ATP and CCh to stimulate Ca^{2+} mobilization depends entirely on their ability to stimulate formation of Ins(1,4,5)P_3, which then causes the Ca^{2+} channel of the Ins(1,4,5)P_3 receptor to open [8].

Previous work established that, during repeated application of a maximal concentration of CCh, there was no desensitization of the signalling pathway leading to Ca^{2+} mobilization [11]; the peak increases in $[Ca^{2+}]_i$ in Ca^{2+}-containing medium were $93 \pm 6\%$ and $94 \pm 8\%$ ($n = 4$) of the initial response after the second and third challenge with CCh (100 μM). The failure of CCh to evoke an increase in $[Ca^{2+}]_i$ after repeated stimulation in Ca^{2+}-free medium (Figure 2A) must therefore result from depletion of the intracellular Ca^{2+} stores available to the Ins(1,4,5)P_3 made in response to activation of muscarinic receptors. Similar results were obtained when the cells were repeatedly stimulated with ATP in Ca^{2+}-free medium (i.e. no response to the second and subsequent stimulation; results not shown), although because the signalling pathway activated by ATP partially desensitizes [11], the loss of response cannot here be unequivocally attributed to depletion of the ATP-sensitive Ca^{2+} stores.

Addition of ATP to cells in which the CCh-sensitive stores had been emptied by prior stimulation in Ca^{2+}-free medium, or of CCh to cells in which the ATP-sensitive stores had been emptied, evoked an increase in $[Ca^{2+}]_i$ that was indistinguishable from that evoked by addition of the same agonist to naïve cells (Figures 2A–2C). Neither the amplitude of the increase in $[Ca^{2+}]_i$ of those cells that responded to the second stimulus (Figure 2C, panel *ii*) nor the fraction of cells that responded (Figure 2C, panel *i*) were affected by prior stimulation with the other agonist.

If even a maximal concentration of one agonist, ATP for example, were incapable of stimulating formation of enough Ins(1,4,5)P_3 to completely empty the Ins(1,4,5)P_3-sensitive Ca^{2+} stores, then a second, more effective, agonist might, by further increasing the intracellular Ins(1,4,5)P_3 concentration, cause Ca^{2+} release from the remaining Ca^{2+} stores. This simple explanation cannot, however, account for our results. First, irrespective of which agonist is used to deplete the Ca^{2+} stores first, the response to the second agonist is undiminished (Figure 2C). Secondly, the amount of Ins(1,4,5)P_3 formed in response to ATP is only about 50% of that stimulated by CCh, and even allowing for the lesser fraction of cells that respond to ATP ($\geqslant 71\%$), the formation of Ins(1,4,5)P_3 in the ATP-responsive cells is still no more than 62% of that evoked by CCh. Yet ATP stimulates Ca^{2+} mobilization after CCh has fully emptied the Ca^{2+} stores to which it has access. Finally, co-application of CCh and ATP stimulates an increase in $[Ca^{2+}]_i$ similar to the sum of the two agonists applied independently (Figure 2C, panel *ii*). We suggest

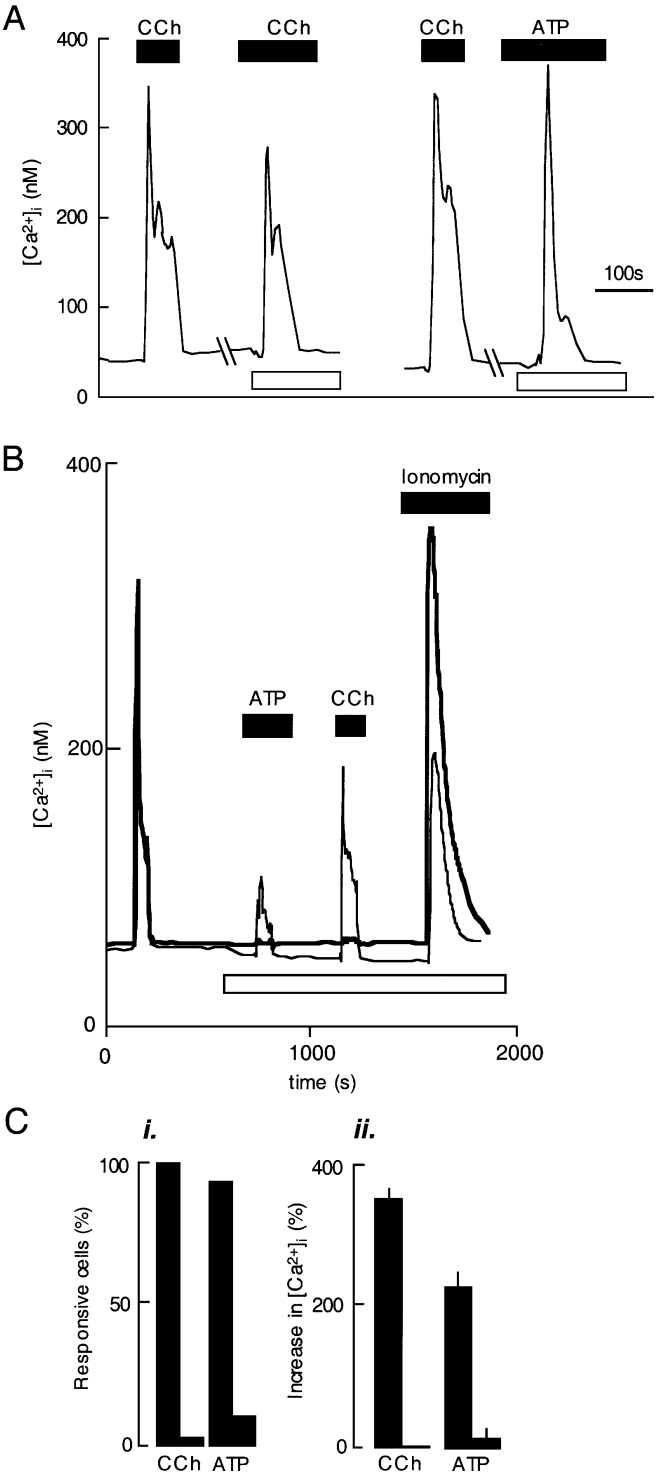

Figure 1 ATP and CCh stimulate Ins(1,4,5)P_3 formation and mobilization of intracellular Ca^{2+} stores

(**A**) Cells were first stimulated with CCh (100 μM; first trace of each pair) in normal ECM to provide the standard response (100%) against which subsequent responses were compared [11]. In Ca^{2+}-free ECM (open horizontal bars), the cells were then stimulated with maximally effective concentrations of CCh (100 μM) or ATP (10 μM). (**B**) In Ca^{2+}-free medium, U73122 (3 μM, applied to the cells shown by the thick trace during the period shown by the open horizontal bar) abolished responses to CCh and ATP, although the intracellular stores could subsequently be released by ionomycin (1 μM). The thin trace shows control responses. The first peak in each trace shows the response to CCh in Ca^{2+}-containing medium. (**C**) From experiments similar to those shown in (**B**), the percentage of cells responding to ATP or CCh (panel *i*) and the peak increase in $[Ca^{2+}]_i$ in those cells that did respond (panel *ii*) are shown for control cells (the first block of each pair) or in the presence of U73122 (3 μM, the second block of each pair). Results are means $\pm$ S.E.M. for at least 37 cells.

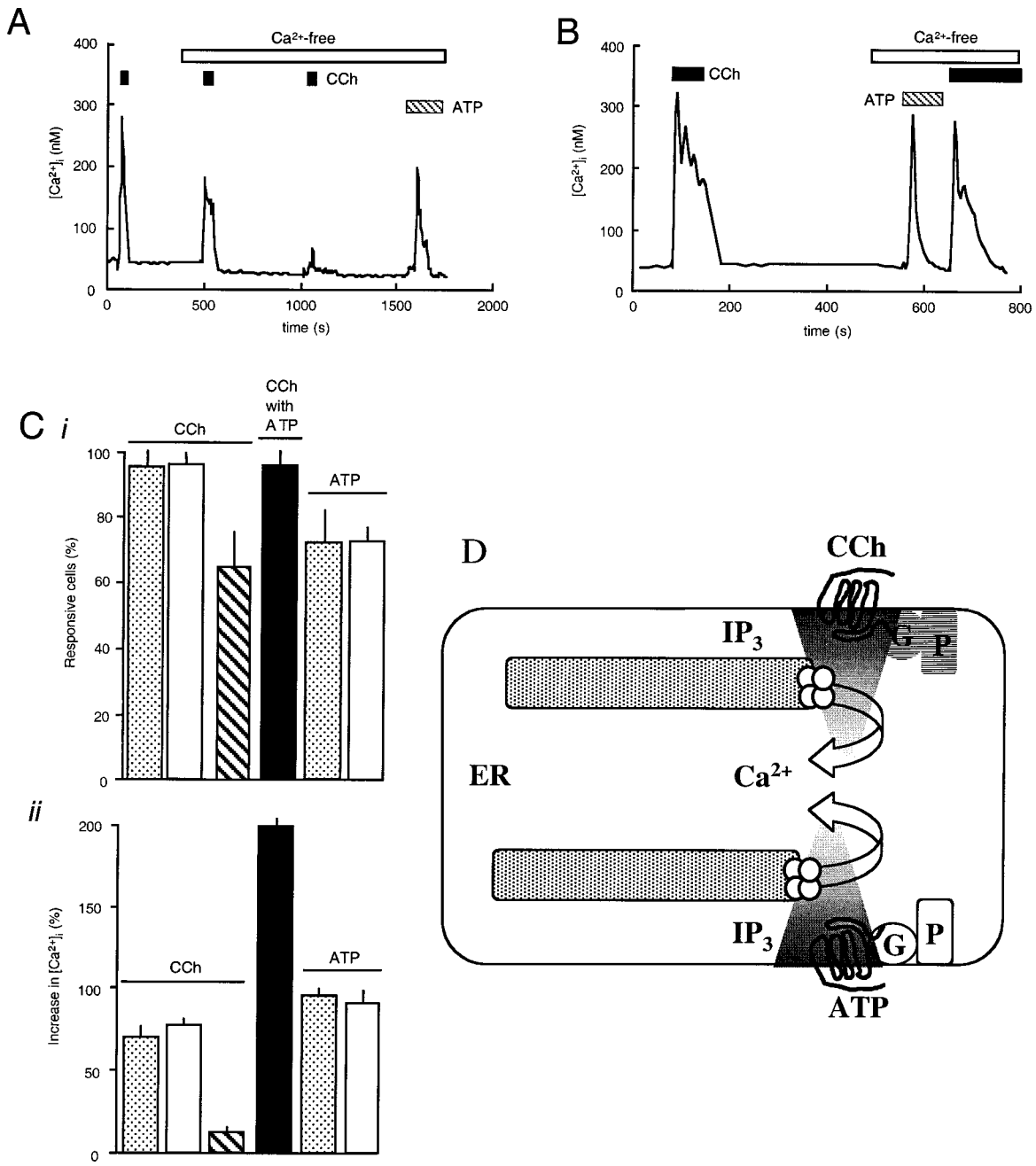

Figure 2 ATP and CCh stimulate mobilization of different intracellular Ca^{2+} pools

(**A** and **B**) After the initial stimulation with CCh (to allow comparison of signals between cells), cells in Ca^{2+}-free ECM were stimulated with maximally effective concentrations of either ATP (10 μM) or CCh (100 μM), before stimulation with a maximal concentration of the other agonist. (**C**) Results from experiments similar to those in (**A**) and (**B**) (means $\pm$ S.E.M., $n = 4$–13 coverslips with $>$ 10 cells analysed on each) are summarized to show both the percentage of cells responding (panel *i*) and the peak amplitude of the increase in $[Ca^{2+}]_i$ in those cells that did respond (panel *ii*). For each panel, stippled blocks denote the responses of naive cells to the stimulus, open blocks the response of the cells after stimulation with the other agonist, and the hatched blocks the response of the cells to the second of two challenges with CCh. The response to simultaneous application of ATP and CCh is shown by the black blocks. (**D**) The two receptors each, via a G protein (G) and phospholipase C (P), stimulate Ins(1,4,5)P_3 ('IP_3') formation, shown as a gradient extending into the cell from the cell-surface signalling molecules. The spatial gradients of Ins(1,4,5)P_3 are proposed to allow each class of cell-surface receptor to stimulate release of Ca^{2+} from different intracellular pools. ER is endoplasmic reticulum.

that, in those cells that respond to both ATP and CCh, both receptors cause Ca^{2+} mobilization by stimulating Ins(1,4,5)P_3 formation and subsequent opening of the Ca^{2+} channel of the Ins(1,4,5)P_3 receptor, but that the Ins(1,4,5)P_3 made in response to each receptor targets different populations of Ins(1,4,5)P_3 receptors (Figure 2D). An analogous situation has been described in polarized epithelia, where purinoceptors at the serosal or mucosal surfaces stimulate Ins(1,4,5)P_3-evoked Ca^{2+} release from different Ca^{2+} stores [13].

There are many examples of cells in which hormones stimulate release of intracellular Ca^{2+} stores without detectable formation of Ins(1,4,5)P_3 [14,15] and others where antagonists of

Ins(1,4,5)P_3 receptors fail to block the Ca^{2+} mobilization evoked by receptors that do stimulate Ins(1,4,5)P_3 formation [16,17]. Such responses may sometimes reflect the involvement of additional Ca^{2+}-mobilizing intracellular messengers [15] such as cADP-ribose, which stimulates ryanodine receptors [18]; nicotinic acid–adenine dinucleotide phosphate (NAADP) [19]; and sphingosine 1-phosphate, which has been shown to cause Ca^{2+} mobilization in HEK-293 cells [20]. These mechanisms are unlikely to explain our results. First, HEK-293 cells do not express ryanodine receptors [20,21]. Secondly, the Ca^{2+} mobilization evoked by maximal stimulation of M3 muscarinic receptors in HEK-293 cells is insensitive to inhibition of the sphingosine kinase responsible for formation of sphingosine 1-phosphate [20]. Thirdly, thapsigargin abolished responses to both ATP and CCh in HEK-293 cells (results not shown), yet the only Ca^{2+} pool so far shown to be released by NAADP was thapsigargin-insensitive [19]. Fourthly, responses to ATP and CCh were abolished when phospholipase C was inhibited (Figures 1B and 1C).

We cannot, of course, eliminate the possibility that, in addition to stimulating Ins(1,4,5)P_3 formation, both ATP and CCh also stimulate formation of an additional co-regulator of Ins(1,4,5)P_3 receptors. In order to explain our observations, however, we would need to propose either that the two agonists caused formation of *different* co-regulating messengers, or else that restricted diffusion of the same messenger allowed the two cell-surface receptors to target the messenger to different Ins(1,4,5)P_3 receptors. An alternative and simpler explanation is consistent with the functional heterogeneity of intracellular Ca^{2+} stores [22] and with studies of pancreatic acinar cells in which different receptors were suggested to preferentially interact with different elements of compartmentalized intracellular Ca^{2+} pools [23]. We suggest that hormone receptors in the plasma membrane and Ins(1,4,5)P_3 receptors are intimately associated, allowing Ins(1,4,5)P_3 to be effectively targetted to only certain Ins(1,4,5)P_3 receptors. A similar situation exists for receptors linked to formation of cAMP, with AKAPs ensuring that cAMP-dependent protein kinase is targetted to allow optimal activation by cyclic AMP [5]. The InaD protein fulfils a similar role in *Drosophila* photoreceptors by anchoring several of the proteins involved in the visual signalling cascade [24]. For Ins(1,4,5)P_3 receptors, targetting of receptors in the plasma membrane may be maintained by proteins analagous to the neuronal Homer proteins that tether metabotropic glutamate receptors to Ins(1,4,5)P_3 receptors [25].

The discovery of cAMP as an intracellular signalling molecule gave rise to the idea that many different receptors regulate cellular activity using a limited repertoire of chemical messengers as a common intracellular currency. Our results suggest that although Ins(1,4,5)P_3 may be an almost ubiquitous intracellular messenger, it may nevertheless allow receptor-specific communication with intracellular Ca^{2+} pools.

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