Coupling of muscarinic receptors to the mobilization of intracellular Ca^{2+} stores in permeabilized SH-SY5Y human neuroblastoma cells

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Intracellular stores of Ca^{2+} were mobilized transiently by carbachol in suspensions of electrically permeabilized SH-SY5Y cells. The kinetics and the dose-dependence of this mobilization paralleled carbachol-induced increases in inositol 1,4,5-trisphosphate (InsP_3) mass [for both parameters EC_{50} (concn. giving half-maximal response) = 60–70 μM]. Guanosine 5'-[γ -thio]triphosphate enhanced the maximal effect and the potency of carbachol on Ca^{2+} mobilization and InsP_3 mass, but caused separation of the dose–response curves (EC_{50} = 0.6 μM and 5.6 μM respectively). These data show that functional coupling of muscarinic receptors to Ca^{2+} mobilization can be maintained after permeabilization, reveal major effects of guanine nucleotides on agonist-induced Ca^{2+} mobilization and provide a basis for explanation of discrepancies between agonist potency on InsP_3 concentration and Ca^{2+} mobilization in intact cells.

INTRODUCTION

It is evident that one of the mechanisms by which extracellular signals are perceived by cells involves activation of cell-surface receptors and consequent generation of inositol 1,4,5-trisphosphate (InsP_3) from phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) hydrolysis (Berridge, 1987). The InsP_3 formed then binds to an intracellular receptor, which in turn causes release of sequestered Ca^{2+} and an increase in intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{free}}$) (Berridge & Irvine, 1989).

The use of permeabilized or disrupted cells has been crucial in establishing and characterizing both the Ca^{2+} -releasing activity of InsP_3 (Berridge, 1987; Nahorski & Potter, 1989) and the involvement of a G-protein, termed G_p , in coupling certain activated receptors to phosphoinositidase C (Taylor & Merritt, 1986; Fain *et al.*, 1988; Birnbaumer *et al.*, 1990). Recently we have considered the possibility that cell-membrane-derived InsP_3 (formed as a consequence of plasma-membrane receptor activation) might, like exogenous InsP_3 , be able to mobilize intracellular stores of Ca^{2+} in permeabilized cells. For these studies we have utilized SH-SY5Y human neuroblastoma cells, which express M3 muscarinic receptors (Lambert *et al.*, 1989), and in which muscarinic agonists stimulate PtdInsP_2 hydrolysis in a guanine-nucleotide-dependent manner after permeabilization (Wojcikiewicz *et al.*, 1990). We report that InsP_3 production and Ca^{2+} release are stimulated by carbachol, acting via muscarinic receptors, and that these effects are enhanced by guanosine 5'-[γ -thio]triphosphate (GTP[S]), a hydrolysis-resistant GTP analogue that binds to and activates G-proteins (Gilman, 1987; Birnbaumer *et al.*, 1990). Thus the coupling apparent in intact SH-SY5Y cells between muscarinic-receptor activation and Ca^{2+} mobilization (Lambert & Nahorski, 1990a) is maintained after cell permeabilization. This has enabled us to conduct a novel investigation of the effect of activation of G_p on the relationship between agonist-induced InsP_3 production and Ca^{2+} release.

METHODS

Ca^{2+} -sensitive electrode

SH-SY5Y cells were cultured, suspended in culture medium and washed (Wojcikiewicz *et al.*, 1990). Cells from one flask were then resuspended in 0.8 ml of ice-cold buffer A (120 mM-KCl, 6 mM- MgCl_2 , 5 mM-sodium succinate, 20 mM-Hepes, 2 mM- KH_2PO_4 , 1 mg of BSA/ml, 5 mM- Na_2ATP , 10 μM quin 2 free acid, pH 7.2) and were exposed to ten discharges of a 3 μF capacitor (Wojcikiewicz *et al.*, 1990). The permeabilized cells were then diluted to 5 ml with buffer A, centrifuged (600 g for 1 min) and finally resuspended in 0.5–1.0 ml of buffer A (~ 4 mg of cell protein/ml). $[\text{Ca}^{2+}]_{\text{free}}$ in the stirred suspension was monitored with a Ca^{2+} -selective electrode (Clapper & Lee, 1985) calibrated by using quin 2 fluorescence. Incubations were performed at 20 $^\circ\text{C}$ to slow the formation and metabolism of InsP_3 , and thus to facilitate accurate measurement of its time-course. Data are expressed as pCa ($-\log [\text{Ca}^{2+}]_{\text{free}}$). $[\text{Ca}^{2+}]_{\text{free}}$ reached a steady state of 0.15–0.3 μM at 10–20 min after resuspension of cells. At this stage small volumes of concentrated solutions of stimuli were added to obtain the final concentrations stated. ATP was gradually consumed during incubations, but > 80% remained after 50 min. For InsP_3 assay, 20 μl samples were removed at various times and quenched with 90 μl of 0.65 M-trichloroacetic acid.

$^{45}\text{Ca}^{2+}$ release

For construction of dose–response curves, incubations with $^{45}\text{Ca}^{2+}$ -prelabelled permeabilized cells were performed. Cells were harvested in 155 mM-NaCl/10 mM-Hepes, pH 7.4 (HBS), plus 0.02% EDTA, centrifuged (500 g for 2 min), resuspended in culture medium, left for 30–60 min at 37 $^\circ\text{C}$, washed twice in HBS and then resuspended in buffer B (buffer A at pH 6.9 without BSA). After 10–30 min at 20 $^\circ\text{C}$, cells were centrifuged, resuspended in buffer B and exposed to three discharges of a 3 μF

Abbreviations used: GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; InsP_3 , inositol 1,4,5-trisphosphate; PtdInsP_2 , phosphatidylinositol 4,5-bisphosphate; G-protein, guanine-nucleotide-binding protein; EC_{50} , agonist concentration causing half-maximal response; $[\text{Ca}^{2+}]_{\text{free}}$, free Ca^{2+} concentration.

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capacitor. The cells were then centrifuged, resuspended (8–16 mg of protein/ml) in buffer B supplemented with oligomycin (2 $\mu\text{g/ml}$) and $^{45}\text{Ca}^{2+}$ (2 $\mu\text{Ci/ml}$) and left to take up $^{45}\text{Ca}^{2+}$ for 15 min at 20 °C. Experimental incubations were started by adding 50 μl of cell suspension to 50 μl of buffer B plus stimuli. After 2 min at 20 °C, silicone oil (Dow Corning 550/556, 9:11, v/v) was added to tubes and the mixture was centrifuged (16000 g for 3 min) to separate cells from buffer before assay of radioactivity in the pellet. Release of $^{45}\text{Ca}^{2+}$ in the presence of stimuli was calculated from the amount remaining in the cell pellet, expressed as a percentage of that sequestered after control incubations (~ 100000 c.p.m. of $^{45}\text{Ca}^{2+}$ /tube). For InsP_3 assay, parallel incubations without $^{45}\text{Ca}^{2+}$ were quenched with 100 μl of 1 M-trichloroacetic acid.

Miscellaneous

InsP_3 concentration was measured by a radioreceptor assay (Challiss *et al.*, 1988). Trichloroacetic acid-quenched samples were centrifuged (16000 g for 5 min) and supernatants (100 μl) were added to 25 μl of 10 mM-EDTA and 100 μl of Freon/octylamine (1:1, v/v). After centrifugation (16000 g for 5 min), upper phases were neutralized with NaHCO_3 and assayed.

EC_{50} values were derived by computer-assisted curve fitting. ATP, GTP and [^3H] InsP_3 levels in cell suspensions were quantified by h.p.l.c., and protein concentration was assayed with Coomassie Blue (Pierce UK Ltd.). Combined data from a number of independent experiments (n) are expressed as means $\pm$ S.E.M. when $n \geq 3$.

Decavanadate was prepared from sodium orthovanadate (Aldrich) as described by Fohr *et al.* (1989). InsP_3 was obtained from Calbiochem; $^{45}\text{CaCl}_2$ was from Amersham International; GTP, GTP[S] and guanosine 5'-[β -thio]diphosphate (GDP[S]) were from Boehringer Mannheim Biochemicals; and Na_2ATP , quin 2 free acid, ionomycin, carbachol and oligomycin were from Sigma.

RESULTS AND DISCUSSION

$[\text{Ca}^{2+}]_{\text{free}}$ in suspensions of electrically permeabilized cells was stimulated transiently by 1 mM-carbachol (Fig. 1a) from 270 ± 20 nM (basal, $n = 6$) to 640 ± 40 nM (peak, $n = 6$) in a manner that was blocked by 10 μM -atropine, a muscarinic antagonist (results not shown). The effects of carbachol were enhanced by 0.1 mM-GTP[S], indicating that a G-protein was involved in mediating the response (Fig. 1b). Thus the signal-transduction pathway, from occupation of cell-surface muscarinic receptors to release of Ca^{2+} from internal stores, was not disrupted by electroporation. Maintenance of this coupling was also seen in SH-SY5Y cells permeabilized with saponin (results not shown), but has been demonstrated only rarely in the past; for example, in 'leaky' pancreatic acinar cells (Streb & Schulz, 1983) and in permeable smooth-muscle cells (Bitar *et al.*, 1986). Thus, although cell permeabilization (particularly that induced by detergents) has been generally considered to decrease the ability of agonists to activate phosphoinositidase C, agonist-stimulated Ca^{2+} mobilization may be common or even universal in permeabilized cell preparations, as long as coupling between receptor and phosphoinositidase C is retained, as is the case in SH-SY5Y cells permeabilized electrically (Wojcikiewicz *et al.*, 1990) or with detergents (Fisher *et al.*, 1989).

That InsP_3 provides the link between receptor activation and Ca^{2+} mobilization in permeabilized SH-SY5Y cells is supported by several findings. Firstly, measurement of InsP_3 mass showed that its concentration correlated well with changes in $[\text{Ca}^{2+}]_{\text{free}}$ (Fig. 1b). As Ca^{2+} is re-sequestered rapidly in cell suspensions as InsP_3 concentration falls (Joseph *et al.*, 1984), the transience of

Fig. 1. Effects of addition of carbachol, GTP[S] and Ca^{2+} on $[\text{Ca}^{2+}]_{\text{free}}$ and InsP_3 concentration

Electrically permeabilized SH-SY5Y cells were resuspended in 0.6–0.9 ml of buffer A (3.0–5.8 mg of cell protein/ml) and, at the times indicated by arrows, 1 mM-carbachol (C), 0.1 mM-GTP[S] (G) and 2.5 μM - InsP_3 (InsP_3) were added (a and b) or 10 nmol of Ca^{2+} /ml (Ca) was added (c). $[\text{Ca}^{2+}]_{\text{free}}$ (continuous line) was monitored with a Ca^{2+} -sensitive electrode, and InsP_3 mass (●) in 20 μl samples of cell suspension was measured as described in the Methods section. $[\text{Ca}^{2+}]_{\text{free}}$ traces are representative of at least three independent experiments, and InsP_3 data are from three (b, means $\pm$ S.E.M.) or two (c, mean $\pm$ range) independent experiments.

Ca^{2+} mobilization can be explained simply by the transience of the increases in InsP_3 concentration (Fig. 1b). Clearly, the post-peak decline in Ca^{2+} mobilization reflected metabolism of neither carbachol nor GTP[S], since further addition of these stimuli did not prevent the decline in $[\text{Ca}^{2+}]_{\text{free}}$ (Figs. 1a and 1b), nor desensitization of the InsP_3 -induced response, since exogenous InsP_3 remained effective when added after the stimuli (Fig. 1b). Secondly, the maximal level of InsP_3 generated, ~ 130 pmol/mg of protein or $0.6 \mu\text{M}$ (Figs. 1b and 2), was sufficient to mobilize Ca^{2+} , as judged from experiments in which exogenous InsP_3 was added. Thirdly, decavanadate (0.1 mM), a putative InsP_3 -receptor antagonist (Fohr *et al.*, 1989), reversed carbachol-plus-GTP[S]-stimulated $^{45}\text{Ca}^{2+}$ release, but did not markedly inhibit stimulation of InsP_3 production (Table 1). InsP_3 -induced $^{45}\text{Ca}^{2+}$ release was similarly reversed, whereas that induced by the Ca^{2+} ionophore ionomycin was unaffected (Table 1). Heparin (M_r 3000), an established InsP_3 -receptor antagonist (Berridge & Irvine, 1989; Nahorski & Potter, 1989), was not used in these studies, as it inhibited InsP_3 -induced Ca^{2+} mobilization only at relatively high concentrations (50% inhibition at $\sim 100 \mu\text{g/ml}$), indicating that its entry into the permeabilized cells was restricted. Finally, the increase in $[\text{Ca}^{2+}]_{\text{free}}$ resulting from addition of carbachol and GTP[S] was equivalent to that obtained upon direct addition of 10–20 nmol of Ca^{2+}/ml (Fig. 1c) or, therefore, release of 2–4 nmol of Ca^{2+}/mg of protein. Addition of 10 nmol of Ca^{2+}/ml raised $[\text{Ca}^{2+}]_{\text{free}}$ to $\sim 1 \mu\text{M}$, but did not significantly alter InsP_3 mass

Fig. 2. Dose-dependence of effects of carbachol on $^{45}\text{Ca}^{2+}$ mobilization and InsP_3 formation

SH-SY5Y cells resuspended in buffer B were exposed to three discharges of a $3 \mu\text{F}$ capacitor and were preincubated with or without $^{45}\text{Ca}^{2+}$ for 15 min at 20°C as described in the Methods section. Experimental incubations with $^{45}\text{Ca}^{2+}$ -labelled cells (for measurement of Ca^{2+} mobilization, $n = 20$, 4–8 mg of protein/ml) and unlabelled cells (for InsP_3 assay, $n = 8$, 3–5 mg of protein/ml) were for 2 min at 20°C . Cells were exposed to a range of carbachol concentrations in the absence ($\circ$, $\square$) or presence ($\bullet$, $\blacksquare$) of 0.1 mM -GTP[S], and $^{45}\text{Ca}^{2+}$ release ($\circ$, $\bullet$) and InsP_3 concentration ($\square$, $\blacksquare$) were assayed. In three additional experiments ($\triangle$, broken line; error bars omitted for clarity), $^{45}\text{Ca}^{2+}$ release was measured in the presence of 1 mM -GDP[S]. Maximal InsP_3 concentration in the presence of carbachol and GTP[S] was approx. 130 pmol/mg of protein ($0.5 \mu\text{M}$).

Table 1. Effects of decavanadate (0.1 mM) on stimulated $^{45}\text{Ca}^{2+}$ mobilization

Experimental incubations with $^{45}\text{Ca}^{2+}$ -labelled electroporated SH-SY5Y cells (4–7 mg of protein/ml) and the additions indicated were performed as described in Fig. 2 ($n \geq 3$). In parallel incubations with cells labelled for 2 days with $[^3\text{H}]\text{inositol}$ (Wojcikiewicz *et al.*, 1990), 0.1 mM -decavanadate decreased carbachol-plus-GTP[S]-stimulated $[^3\text{H}]\text{InsP}_3$ formation by only 9.5% (mean, $n = 2$).

	$^{45}\text{Ca}^{2+}$ release (% of control)	
	No decavanadate	+decavanadate
Control	0	2 ± 2
$10 \mu\text{M}$ -Carbachol + 0.1 mM -GTP[S]	33 ± 6	6 ± 2
$1 \mu\text{M}$ - InsP_3	32 ± 5	5 ± 3
$1 \mu\text{M}$ -Ionomycin	56 ± 6	55 ± 9

(Fig. 1c). Thus the increase in InsP_3 concentration causes Ca^{2+} mobilization rather than the converse.

The enhancement by GTP[S] of the effects of carbachol was examined further by using electroporated cells pre-labelled with $^{45}\text{Ca}^{2+}$ (Fig. 2). Carbachol alone maximally stimulated release of $32 \pm 2\%$ of sequestered $^{45}\text{Ca}^{2+}$ ($\text{EC}_{50} = 70 \pm 12 \mu\text{M}$, $n = 20$). GTP[S] alone had little effect on $^{45}\text{Ca}^{2+}$ mobilization, but enhanced both the maximal effect ($41 \pm 2\%$) and potency of carbachol ($\text{EC}_{50} = 0.6 \pm 0.3 \mu\text{M}$, $n = 20$). In contrast, GDP[S], which antagonizes activation of G_p and thus receptor-mediated PtdInsP_2 breakdown (Taylor & Merritt, 1986; Fain *et al.*, 1988; Wojcikiewicz *et al.*, 1990), blocked the effect of carbachol (Fig. 2). These actions of GDP[S] and the ability of carbachol to stimulate in the absence of added guanine nucleotide imply that sufficient endogenous GTP was present to enable G_p to fulfil, at least partially, its role as a transducer of receptor activation. Indeed, chromatographic analysis identified $\sim 50 \mu\text{M}$ -GTP in cell suspensions (results not shown).

Under these conditions, exogenous InsP_3 released maximally $47 \pm 2\%$ of $^{45}\text{Ca}^{2+}$ ($\text{EC}_{50} = 0.26 \pm 0.08 \mu\text{M}$, $n = 6$). Although it has been proposed that GTP facilitates InsP_3 -induced Ca^{2+} release by enlarging the InsP_3 -sensitive Ca^{2+} store in a manner independent of signal-transducing G proteins but inhibitable by GTP[S] and GDP[S] (Gill *et al.*, 1988), such a mechanism was not apparent in the present study, as neither maximal release nor EC_{50} for InsP_3 was significantly altered by 1 mM -GDP[S], 0.1 mM -GTP[S] or by additional GTP (results not shown).

Measurement of InsP_3 mass in parallel incubations with unlabelled cells enabled the relationship between agonist-induced InsP_3 production and $^{45}\text{Ca}^{2+}$ mobilization to be examined. In the absence of GTP[S], carbachol-stimulated $^{45}\text{Ca}^{2+}$ mobilization ($\text{EC}_{50} = 70 \pm 12 \mu\text{M}$) reflected closely changes in InsP_3 concentration ($\text{EC}_{50} = 60 \pm 19 \mu\text{M}$, $n = 8$), indicating that the two parameters were related in direct proportion. In contrast, in the presence of GTP[S], the dose-response curve for $^{45}\text{Ca}^{2+}$ release ($\text{EC}_{50} = 0.6 \pm 0.3 \mu\text{M}$) lay to the left of that for InsP_3 ($\text{EC}_{50} = 5.6 \pm 1.8 \mu\text{M}$, $n = 8$).

This effect of GTP[S] on InsP_3 production is consistent with our recent findings that GTP[S], by virtue of its ability persistently to activate G-proteins, including G_p , modifies the relationship between occupation of the muscarinic receptor and the phosphoinositide response and that, in effect, GTP[S] enhances the efficiency of coupling between receptor and phosphoinositidase C (Wojcikiewicz *et al.*, 1990). Thus, in the present study, GTP[S] caused a 67% increase in maximal InsP_3 concentration (from $0.3 \mu\text{M}$ to $0.5 \mu\text{M}$) and an 11-fold decrease in the EC_{50} of carbachol. As would be expected, $^{45}\text{Ca}^{2+}$ mobilization followed the same

pattern, although the EC_{50} of carbachol decreased to a greater extent (117-fold) and the increase in maximal effect was only 28%. This loss of proportionality between $^{45}Ca^{2+}$ release and $InsP_3$ concentration in the presence of GTP[S] can be accounted for, in part, by a limit on the extent to which sequestered $^{45}Ca^{2+}$ can be mobilized by $InsP_3$ (exogenous $InsP_3$ released maximally only $47 \pm 2\%$ of sequestered $^{45}Ca^{2+}$) and the generation of concentrations of $InsP_3$ greater than that required to give maximal Ca^{2+} mobilization. Thus, in the presence of GTP[S], when maximal $InsP_3$ concentration was $\sim 0.5 \mu M$ and the amount of $^{45}Ca^{2+}$ mobilized (41%) was close to the maximum amount possible, the $^{45}Ca^{2+}$ -release curve was truncated and the EC_{50} for carbachol on $^{45}Ca^{2+}$ release was decreased relative to that for $InsP_3$ concentration. In contrast, in the absence of GTP[S], maximal carbachol-induced $InsP_3$ concentration ($0.3 \mu M$) was sufficient to release only 32% of $^{45}Ca^{2+}$, and thus the limit on $^{45}Ca^{2+}$ mobilization was not approached. This explanation, however, does not account for the observation in Fig. 2 that a given concentration of plasma-membrane-derived $InsP_3$, for example $0.15 \mu M$ (30% of maximal response), appears to cause more $^{45}Ca^{2+}$ release in the presence of GTP[S] than in its absence. It seems likely that this discrepancy relates to differences between the estimates of $InsP_3$ concentration (reflecting a balance of $InsP_3$ synthesis and degradation 2 min after addition of stimuli) and the initial rates of $InsP_3$ generation, which may be more important in influencing the amount of $^{45}Ca^{2+}$ release.

These findings appear to pertain to the situation in intact SH-SY5Y cells in which separation of the dose-response curves is also evident; the EC_{50} value for carbachol on inositol phosphate formation is $17\text{--}31 \mu M$ (Serra *et al.*, 1988; Wojcikiewicz *et al.*, 1990; Lambert & Nahorski, 1990b) and that on $InsP_3$ mass is $\sim 40 \mu M$ (D. G. Lambert & S. R. Nahorski, unpublished work), whereas that for the initial phase of Ca^{2+} mobilization is $4\text{--}8 \mu M$ (Lambert & Nahorski, 1990a; Akerman, 1989). Similarly, the EC_{50} for vasopressin-induced Ca^{2+} mobilization is 25-fold less than that for $InsP_3$ production in isolated rat hepatocytes (Thomas *et al.*, 1984). The slight separation of the dose-response curves seen in intact SH-SY5Y cells is either accentuated or diminished in the present study with permeabilized cells, depending on whether or not GTP[S] is present. This appears to be because GTP[S] persistently activates G_p , and thus leads to marked enhancement of carbachol effects and accumulation of relatively high levels of $InsP_3$. In contrast, in the absence of GTP[S], GTP supports G_p activity, and the potency and efficacy of carbachol on $InsP_3$ production are decreased relative to that seen in intact cells (Wojcikiewicz *et al.*, 1990).

In conclusion, the coupling between muscarinic-receptor activation, $InsP_3$ production and Ca^{2+} mobilization seen in intact SH-SY5Y cells (Lambert & Nahorski, 1990a) is maintained after

cell permeabilization. Generation of $InsP_3$ concentrations above those required to produce maximal Ca^{2+} mobilization accounts, in part, for the loss of proportionality between these parameters in the presence of GTP[S], and provides an explanation for the discrepancies seen in intact cells between agonist potency on $InsP_3$ generation and Ca^{2+} mobilization. Further studies with systems such as that described here, in which signalling processes are particularly vulnerable to perturbation and regulation by exogenous factors, should greatly assist our understanding of the molecular events that mediate receptor-activated Ca^{2+} mobilization.

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