Muscarinic-receptor-mediated changes in intracellular Ca^{2+} and inositol 1,4,5-trisphosphate mass in a human neuroblastoma cell line, SH-SY5Y

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This study reports increased intracellular Ca^{2+} and inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] in response to muscarinic-cholinergic stimulation of human neuroblastoma (SH-SY5Y) cells. Carbachol stimulation leads to a rapid increase in intracellular Ca^{2+} and $\text{Ins}(1,4,5)\text{P}_3$ mass, both reaching a peak at around 10 s and then declining to a new maintained phase significantly above basal. Dose-response analysis of peak and plateau phases of intracellular Ca^{2+} shows different agonist potencies for both phases, carbachol being more potent for the plateau phase. The plateau-phase intracellular Ca^{2+} was dependent on extracellular Ca^{2+} , which is admitted to the cell through a non-voltage-sensitive Ni^{2+} -blockable Ca^{2+} channel. Using a Mn^{2+} quench protocol, we have shown that Ca^{2+} entry occurs early during the discharge of the internal stores. The plateau phase (Ca^{2+} -channel opening) is dependent on the continued presence of agonist, since addition of atropine closes the Ca^{2+} channel and intracellular Ca^{2+} declines rapidly back to basal. We also failed to detect a refilling transient when we added back Ca^{2+} after intracellular Ca^{2+} had reached a peak and then declined in Ca^{2+} -free conditions. These data strongly suggest that muscarinic stimulation of SH-SY5Y cells leads to a rapid release of Ca^{2+} from an $\text{Ins}(1,4,5)\text{P}_3$ -sensitive internal store and a parallel early entry of Ca^{2+} across the plasma membrane.

INTRODUCTION

Changes in neuronal cytoplasmic free Ca^{2+} are intimately associated with cell survival (Choi, 1988), growth (Kater *et al.*, 1988) and transmitter release (Smith & Augustine, 1988). Intracellular Ca^{2+} is tightly regulated to a resting level of some 100 nM by a combination of binding to Ca^{2+} -specific binding proteins, sequestration into intracellular pools, active extrusion from the cytosol by an energy-requiring Ca^{2+} pump mechanism, and through increased activity of the $\text{Na}^+/\text{Ca}^{2+}$ antiport system (Blaustein, 1988). The two most extensively characterized routes by which agonists increase intracellular Ca^{2+} are the release of Ca^{2+} from an intracellular store, generally believed to be the endoplasmic reticulum and/or the specialized calciosome (Streb *et al.*, 1983; Volpe *et al.*, 1988) and the opening of membrane Ca^{2+} channels (Meldolesi & Pozzan, 1987). Agents that induce an increase in phosphoinositide hydrolysis and generation of the second messenger $\text{Ins}(1,4,5)\text{P}_3$ cause a rapid transient release of intracellular bound Ca^{2+} into the cytoplasm in many cells (Berridge, 1987), including neurons (Nahorski, 1988). The opening of Ca^{2+} channels is more complex. In neuronal tissue there are at least three distinct forms of voltage-gated Ca^{2+} channels, namely the T, L and N channels (Tsien *et al.*, 1988). There is also growing evidence for the existence of two other forms of Ca^{2+} channels, the so-called SMOCC (second-messenger-operated Ca^{2+} channel), and the ROCC (receptor-operated Ca^{2+} channel) (Meldolesi & Pozzan, 1987; Penner *et al.*, 1988).

Our understanding of the mechanisms of intracellular Ca^{2+} homeostasis have been greatly assisted by the use

of fluorescent dyes such as fura2 (Cobbold & Rink, 1987). There have been several studies in which agonist-stimulated increases in intracellular Ca^{2+} , monitored by such dyes, have been shown to be biphasic. These include carbachol-stimulated parotid and thrombin-stimulated platelets (Merritt & Hallam, 1988), carbachol-stimulated parietal cells (Negulescu & Machen, 1988), thrombin- and histamine-stimulated human endothelial cells (Hallam *et al.*, 1988) and bradykinin-stimulated NG115-40L neuroblastoma-glioma hybrids (Jackson *et al.*, 1987). In all of these studies, removal of extracellular Ca^{2+} converts the biphasic Ca^{2+} signal into a simple monophasic response. Furthermore, as shown by Hallam & Rink (1985), entry of Ca^{2+} across the plasma membrane can be monitored by using the bivalent cation Mn^{2+} , which quenches fura2 fluorescence at all excitation wavelengths. Since there are no intracellular stores of Mn^{2+} , agonist-stimulated quenching is indicative of agonist-stimulated bivalent-cation entry. Thus several agonists that induce a biphasic increase in intracellular Ca^{2+} also stimulate the production of $\text{Ins}(1,4,5)\text{P}_3$, the consequent release of intracellular Ca^{2+} and the opening of a Ca^{2+} channel. There has been much speculation as to the relationship between these cellular events (Irvine *et al.*, 1988; Nahorski, 1988; Fasolato *et al.*, 1988).

Here we examine the cholinergic regulation of intracellular Ca^{2+} and increased $\text{Ins}(1,4,5)\text{P}_3$ mass in the human neuroblastoma cell line SH-SY5Y, apparently possessing, on pharmacological grounds, a homogeneous M3 muscarinic-receptor complement (Lambert *et al.*, 1989) and a marked phosphoinositide response to muscarinic agonists (Lambert & Nahorski, 1989; Wojcikiewicz *et al.*, 1990). The present studies reveal a

Abbreviation used: EC_{50} , concn. giving half-maximal stimulation.

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biphasic increase in intracellular Ca^{2+} with different agonist potencies for the peak and maintained phases. We present further data showing that the maintained phase is dependent on agonist occupation of the receptor and on extracellular Ca^{2+} , and that Ca^{2+} entry occurs at early time points when the intracellular stores are only partially emptied.

MATERIALS AND METHODS

Sources of reagents

Reagents of analytical grade and double-distilled water were used throughout. The chemicals and their sources (U.K. unless otherwise stated) were as follows. Minimum essential medium, trypsin/EDTA, foetal-calf serum, glutamine, penicillin/streptomycin, fungizone and 175 cm^2 tissue-culture flasks were from Gibco. Fura2/AM (penta-acetoxymethyl-ester; lot 695000) was from Calbiochem (La Jolla, CA, U.S.A.). Atropine, arecoline, carbachol, muscarine, verapamil and Triton X-100 were from Sigma Chemical Co. +PN 200-110 was from Sandoz AG (Basel, Switzerland). Ω -Conotoxin was from Peptide Institute Inc. (Osaka, Japan). D/L-Ins(1,4,5) P_3 was provided by Dr B. V. L. Potter (Department of Chemistry, University of Leicester), D-Ins(1,4,5) P_3 was from Amersham, and D-[^3H]Ins(1,4,5) P_3 (17 Ci/mmol) was from NEN. Freon (1,1,2-trichloro-1,2,2-trifluoroethane) was from Fisons Scientific, and tri-n-octylamine was from Aldrich Chemicals. All other reagents were from BDH.

Cell culture

SH-SY5Y human neuroblastoma stock cultures (kindly given by Dr J. Biedler, Sloan-Kettering Institute, New York, NY, U.S.A.) were routinely maintained in minimum essential medium supplemented with 2 mM-L-glutamine, 100 i.u. of penicillin/ml, 100 μg of streptomycin/ml, 2.5 μg of fungizone/ml and 10% (v/v) foetal-calf serum. Cultures were seeded into 175 cm^2 tissue-culture flasks containing 30 ml of supplemented medium and maintained at 37 °C in 5% CO_2 /humidified air. Stock cultures were passaged at 1:3 weekly and fed twice weekly. All experimental work reported here was performed with cells from passages 62–90.

Measurement of intracellular Ca^{2+}

Confluent 6–7 day cultures of SH-SY5Y cells were scraped from the tissue-culture flasks into Krebs buffer of the following composition (mM): Na^+ 143.3, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 1.3, Cl^- 128.3, $\text{H}_2\text{PO}_4^{2-}$ 2.2, HCO_3^- 24.9, SO_4^{2-} 1.2 and glucose 10. After two washes in fresh Krebs buffer the suspension was incubated for 45 min at 37 °C with 5 μM -fura2/AM. At the end of this 'loading' period, the cell suspension was washed three times in Krebs buffer and resuspended in an appropriate volume (approx. 18–24 ml/flask) of fresh buffer. The stock of loaded cells was maintained at room temperature until use. This procedure has been shown to reduce fura2 leakage (Malgaroli *et al.*, 1987).

Intracellular Ca^{2+} was measured in 3 ml suspensions of fura-2-loaded SH-SY5Y cells at 37 °C in polypropylene cuvettes containing a stirrer bar. Fura2 fluorescence was monitored in a Perkin-Elmer LS5B spectrofluorimeter. The excitation wavelengths were 340 and 380 nm (in some experiments 340 and the isobestic point of fura2, 360 nm, were used), with emission at 509 nm. The time

taken to drive between 340 and 380 nm excitation intensities was 3.8 s. Intracellular Ca^{2+} was calculated from the ratio of fluorescence at 340/380 nm excitation wavelengths as described by Grynkiewicz *et al.* (1985), where R_{max} and R_{min} were determined with Triton X-100 (0.1%) and EGTA (3 mM) respectively. Where Ca^{2+} -free conditions were required, fura2 loaded cells were washed and resuspended in nominally Ca^{2+} -free Krebs buffer.

Measurement of D-Ins(1,4,5) P_3 mass

This was done by a radioreceptor method previously described and evaluated for stereo- and positional specificity (Challiss *et al.*, 1988). Briefly, intact SH-SY5Y cells (0.7–1.4 mg) were preincubated in Krebs buffer for 5 min at 37 °C. Carbachol (1 mM) was then added for the indicated time periods. The reaction was stopped with an equal volume of trichloroacetic acid (1 M). D-Ins(1,4,5) P_3 was extracted with Freon/tri-n-octylamine (1:1, v/v) and neutralized with 25 mM- HCO_3^- . D-Ins(1,4,5) P_3 was quantified by radioreceptor assay using a bovine adrenal-cortical binding protein at 4 °C. Authentic D-Ins(1,4,5) P_3 (0.036–36 pmol) made in buffer, taken through an identical extraction, was used as a standard, and D-[^3H]Ins(1,4,5) P_3 as a radioligand. Non-specific binding was defined in the presence of excess D/L-Ins(1,4,5) P_3 (0.3 nmol). Bound D-[^3H]Ins(1,4,5) P_3 was separated by filtration and quantified by liquid-scintillation spectrometry.

Data analysis

Data are expressed as means $\pm$ S.E.M. of at least three determinations. EC_{50} (half-maximum stimulation) values were obtained by computer-assisted curve fitting using ALLFIT (De Lean *et al.*, 1978). Where appropriate, statistical significance was assessed by Student's *t* test and was considered significant when $P < 0.05$.

RESULTS

Carbachol causes a dose-related increase in intracellular Ca^{2+} which is biphasic at agonist concentrations down to 10 μM (Fig. 1). At 1 mM-carbachol intracellular Ca^{2+} rises rapidly from a resting value of 70 ± 8 nM to 615 ± 62 nM some 8.7 ± 1.2 s (mean $\pm$ S.E.M., $n = 3$) after agonist addition; this peak then declined to a new steady plateau level some 30–60 s later. The plateau level was elevated above basal and persisted for at least 6 min, during which time further agonist addition had no effect (results not shown). The dose/response relationship to the production of the initial spike of intracellular Ca^{2+} and the maintained plateau phase are shown in Fig. 2. Also shown are the effects of the muscarinic agonists muscarine and arecoline. EC_{50} values obtained by computer-assisted curve fitting (Table 1) show that carbachol and muscarine have similar affinities for both spike and plateau and, more importantly, that there is at least a 10-fold difference between spike and plateau phases for both agonists. Arecoline only consistently produced a spike of intracellular Ca^{2+} , which was 5-fold lower than that produced by carbachol and muscarine at an agonist concentration of 1 mM. At 0.1 mM-arecoline an intracellular Ca^{2+} spike was observed in two of three experiments. The EC_{50} value for the arecoline-stimulated plateau phase did not differ from that seen during the initial spike phase observed with carbachol and mus-

Fig. 1. Effect of carbachol (arrow) on intracellular Ca^{2+} in fura2-loaded human neuroblastoma cells

[Carbachol] added: (a) 1 mM; (b) 100 μ M; (c) 10 μ M; (d) 1 μ M; (e) 0.1 μ M. Intracellular Ca^{2+} was monitored at 37 $^{\circ}$ C, and data shown are from a single experiment typical of two others. Data points were taken every 3.8 s.

carine (Table 1). Nevertheless there still appears to be a large separation between spike and plateau phases with this weaker partial agonist. Preincubation of the cells with the muscarinic antagonist atropine suppressed both components of the Ca^{2+} signal (see below).

Addition of 1 mM-carbachol to SH-SY5Y cells caused a rapid increase in D- $Ins(1,4,5)P_3$ mass. Basal values of 29.4 ± 7.8 pmol/mg of protein increased to 466.4 ± 141.8 pmol/mg after 10 s ($n = 5$), and declined more slowly until by 1 min a new steady state, increased significantly ($P < 0.05$) above basal, was reached. In view of the variability between experiments, data are expressed as percentages of maximum response, but in all experiments there was at least a 6.3-fold stimulation (Fig. 3).

When SH-SY5Y cells are stimulated with 1 mM-carbachol in the absence of extracellular Ca^{2+} (+0.1 mM-EGTA), the biphasic intracellular Ca^{2+} response is converted into a simple monophasic profile, intracellular Ca^{2+} reaching a peak and then returning to basal values (Fig. 4). In the absence of extracellular Ca^{2+} , the spike phase is decreased by an amount equivalent to the level of the normal plateau phase seen in its presence. The level of the plateau phase is not affected by prior preincubation with the voltage-sensitive Ca^{2+} -channel antagonists verapamil (1 μ M), +PN 200-110 (1 μ M) and Ω -conotoxin (0.1 μ M), but was suppressed by $NiCl_2$ (5 mM; results not shown). In parallel experiments both +PN 200-110 and Ω -conotoxin decreased K^+ (30 and 60 mM)-stimulated intracellular Ca^{2+} , Ni^{2+} completely abolished the intracellular- Ca^{2+} response to K^+ , whereas Ni^{2+} converted the biphasic intracellular- Ca^{2+} response

to carbachol into a monophasic profile (D. G. Lambert, E. M. Whitham, J. G. Baird & S. R. Nahorski, unpublished work). If Ca^{2+} (1.2 mM) is replaced to cells previously stimulated (0.1 mM-carbachol) under Ca^{2+} -free conditions, then the plateau phase returns, and the sum of the peak and plateau intracellular Ca^{2+} values is roughly equivalent to the normal intracellular Ca^{2+} response to carbachol (Figs. 5a and 5b). If the muscarinic receptor is blocked with atropine (1 μ M) after the spike of intracellular Ca^{2+} has occurred and extracellular Ca^{2+} is then added back, a small increase in intracellular Ca^{2+} is seen (Fig. 5c). This small increase is due to a re-adjustment of basal Ca^{2+} or to fura2 leakage from the cells, since addition of water instead of carbachol produces an identical increase (Fig. 5d).

Using the regimen described in Fig. 5, namely inducing an agonist-induced Ca^{2+} spike in the absence of extracellular Ca^{2+} and then adding back Ca^{2+} to obtain the plateau phase, we have re-evaluated the dose/response relationship of spike and plateau intracellular Ca^{2+} . Fig. 6 shows a dose-dependent increase in intracellular Ca^{2+} during both spike and plateau phases, with corresponding EC_{50} values of 11.6 ± 4.1 μ M and 2.1 ± 0.8 μ M ($P < 0.05$), confirming the difference in carbachol potencies for spike and plateau shown in Table 1.

As indicated in Fig. 4, removal of extracellular Ca^{2+} eliminates the plateau phase of intracellular Ca^{2+} . We have further examined the influence of extracellular Ca^{2+} on the plateau phase of intracellular Ca^{2+} by replacing extracellular Ca^{2+} with 0.1 mM- Mn^{2+} (Mn^{2+} quenches fura2 fluorescence at all excitation intensities). Fig. 7(a)

Fig. 2. Dose/response profiles of carbachol, muscarine and arecoline for spike ($\square$) and plateau ($\blacklozenge$) phases of intracellular Ca^{2+} in fura2-loaded human neuroblastoma cells.

Data are means $\pm$ S.E.M. of three experiments (* $P < 0.05$ compared with corresponding plateau).

shows raw fluorescence data at 340 and 380 nm excitation intensities in SH-SY5Y cells stimulated with 0.1 mM-carbachol in the absence of added Ca^{2+} . Addition of carbachol causes a rapid increase in fluorescence at 340 nm, which then returns to basal. At 380 nm the fluorescence declines and then returns back up to basal levels. Subsequent addition of 0.1 mM- Mn^{2+} caused rapid quenching of fura2 fluorescence at both excitation wavelengths. If Mn^{2+} is added before carbachol (Fig. 7b), then we see an increase in fluorescence at 340 nm, which declines to significantly below basal levels. Fluorescence at 380 nm declines and, owing to quenching, is no longer able to return to pre-stimulus values. If this experiment is repeated (Fig. 7c) using 340 nm and 360 nm excitation

Table 1. EC_{50} values for muscarinic-agonist-induced biphasic increases in intracellular free Ca^{2+} concentration of fura2-loaded SH-SY5Y human neuroblastoma cells

Data are means $\pm$ S.E.M. of three independent experiments.

Agonist	EC_{50} (μM)	
	Spike	Plateau
Carbachol	7.62 ± 2.53	$0.65 \pm 0.25^*$
Muscarine	7.54 ± 2.17	$0.42 \pm 0.15^*$
Arecoline	> 100	$7.15 \pm 2.58^{\dagger\dagger}$

* $P < 0.05$ compared with spike.

$\dagger$ $P < 0.05$ compared with carbachol and arecoline plateau.

$\ddagger$ $P > 0.05$ compared with carbachol and arecoline spike.

Fig. 3. Time course for the production of $\text{D-Ins}(1,4,5)\text{P}_3$ in carbachol (1 mM)-stimulated SH-SY5Y cells.

Reactions were terminated with 1 M-trichloroacetic acid, and $\text{D-Ins}(1,4,5)\text{P}_3$ was measured by radioreceptor assay as described in the Materials and methods section. Data represent means $\pm$ S.E.M. ($n = 4-5$). Basal values were 29.4 ± 7.8 pmol/mg of protein, rising to 466.4 ± 141.8 pmol/mg at 10 s (* $P < 0.05$ compared with zero time). Key: $\square$, + Carbachol; $\blacklozenge$, no carbachol.

intensities (the latter being the isosbestic point for fura2 that is insensitive to changes in Ca^{2+}), a similar pattern to that at 340 nm is seen. However, at 360 nm quenching, indicative of Mn^{2+} entry, is clearly occurring at early time points during the spike of fluorescence observed at 340 nm.

Fig. 4. Removal of extracellular Ca^{2+} blocks the plateau phase of intracellular Ca^{2+} in fura2-loaded human neuroblastoma cells.

Cells were either incubated in the presence of external Ca^{2+} (□) or washed in Ca^{2+} -free buffer and 0.1 mM-EGTA was added 1 min before carbachol (1 mM) addition (arrow) (◆). Intracellular Ca^{2+} was monitored at 37 °C, and data shown are from a single experiment typical of two others. Data points were taken every 3.8 s.

DISCUSSION

This study has clearly demonstrated the biphasic nature of the muscarinic-agonist-stimulated increase in intracellular Ca^{2+} of SH-SY5Y human neuroblastoma cells. This pattern of Ca^{2+} change, with an initial short-lived spike response, followed by a prolonged plateau (which is dependent on extracellular Ca^{2+}), resembles that seen in several other cell types with various agonists (Merritt & Hallam, 1988; Negulescu & Machen, 1988; Hallam *et al.*, 1988; Jackson *et al.*, 1987).

There have been two recent reports on Ca^{2+} signalling in this clone of neuroblastoma cells. Heikkilä *et al.* (1987) reported a small biphasic increase in quin2 fluorescence after cholinergic agonists that was substantially decreased in the absence of extracellular Ca^{2+} . Akerman (1989), on the other hand, reported a larger but monophasic fura2 signal after carbachol stimulation of these cells. It is difficult to offer a reasoned explanation for differences between these studies and with the present sustained biphasic Ca^{2+} signals induced by muscarinic agonists. Merritt & Rink (1987a,b) have shown that in parotid acinar cells both substance P and carbachol respectively display different EC_{50} values for spike and plateau phases of intracellular Ca^{2+} . They concluded that lower EC_{50} of carbachol for the plateau phase, which is similar to its

binding affinity in the parotid (Putney & Van de Walle, 1980), may reflect a process more closely linked to receptor occupation with fewer amplification processes. The EC_{50} values for spike and plateau observed here by using the regimen in Fig. 5 revealed a somewhat smaller difference in EC_{50} values of only 5.5-fold, whereas when the direct approach was used, as in Figs. 1 and 2, the difference was some 11.7-fold. This discrepancy may result from measurements taken at the peak of intracellular Ca^{2+} (Figs. 1 and 2) involving some of the plateau increase in intracellular Ca^{2+} , i.e. the Ca^{2+} channel is opening during the discharge of the intracellular stores (see below).

It is tempting to suggest that the initial spike is mediated by the second messenger $Ins(1,4,5)P_3$. We have shown in these cells that the increase in intracellular Ca^{2+} seen during the spike phase after carbachol stimulation is paralleled by a dramatic increase in the mass of $Ins(1,4,5)P_3$ measured by a radioreceptor assay. These experiments are important, since, in earlier studies using [3H]inositol-labelled cells, relative changes in [3H] $InsP_3$ separated by conventional anion-exchange chromatography and [3H] $Ins(1,4,5)P_3$ separated on h.p.l.c. were small, perhaps indicating that other labelled triphosphates are present in control cells. The basal mass level of $Ins(1,4,5)P_3$ in SH-SY5Y cells is relatively high, but closely resembles those recently found in brain (Challiss *et al.*, 1988) and bovine tracheal smooth-muscle slices (Chilvers *et al.*, 1989) by an identical assay. Indeed, resting $Ins(1,4,5)P_3$ in NG108 and NIH3T3 cells has also been reported in the same range (Fu *et al.*, 1988), and indicates that, if homogeneously distributed in the cell, concentrations $> 1 \mu M$ would be expected. The specificity of the assay has been carefully established both with a variety of inositol polyphosphates and by the sensitivity of assayable material to 5-phosphatase and 3-kinase activity (R. A. J. Challiss, E. R. Chilvers & S. R. Nahorski, unpublished work). Since $Ins(1,4,5)P_3$ releases intracellular Ca^{2+} from permeabilized SH-SY5Y cells at sub-micromolar concentrations (Nahorski & Potter, 1989; S. T. Safrany & S. R. Nahorski, unpublished work), substantial compartmentalization of this second messenger in resting cells must be expected. At present we do not know with certainty why $InsP_3$ levels remain elevated above basal for such protracted periods (> 300 s). This elevated $InsP_3$ does not appear to release Ca^{2+} , possibly owing to receptor desensitization or relatively slow movement of Ca^{2+} between $InsP_3$ -sensitive and non-sensitive pools. Fisher *et al.* (1989) have shown in the parent cell line that removal of extracellular Ca^{2+} decreases production of labelled inositol polyphosphates, perhaps suggesting that the maintained elevation in $InsP_3$ observed in the present study is secondary to Ca^{2+} entry.

The maintenance of the plateau phase is dependent on extracellular Ca^{2+} and is apparently controlled by the opening of a non-voltage-sensitive Ni^{2+} -blockable Ca^{2+} channel. The decreased ability of arecoline to produce this biphasic Ca^{2+} response may relate to the poorer coupling of the receptor to phospholipase C and Ca^{2+} -channel opening induced by this partial agonist. Certainly, in these cells arecoline is only 45% as efficacious as carbachol on the production of [3H]inositol phosphates (Wojcikiewicz & Nahorski, 1989; Wojcikiewicz *et al.*, 1990).

A more thorough investigation of the nature of the plateau phase revealed its dependence on extracellular

Fig. 5 Maintenance of the plateau phase of intracellular Ca^{2+} is dependent on the continued presence of agonist

Panel (a) shows a normal biphasic intracellular Ca^{2+} profile in response to 0.1 mM-carbachol (CCH) in the presence of external Ca^{2+} . When external Ca^{2+} is removed, the plateau is removed, but re-addition of Ca^{2+} (1.2 mM) restores the plateau phase (b). The agonist-dependency of the plateau phase is shown in panels (c) and (d), in which carbachol is removed from its receptor with atropine (1 μM) (c) and carbachol is replaced with water (d). Intracellular Ca^{2+} was monitored at 37 $^{\circ}\text{C}$ and data shown are from a single experiment typical of two others. Data points were taken every 3.8 s.

Fig. 6 Dose-related increase in intracellular Ca^{2+} during the spike and plateau phases after agonist addition

Data for the spike of intracellular Ca^{2+} ($\square$) were obtained in Ca^{2+} -free buffer. Data for the plateau phase of intracellular Ca^{2+} ($\blacklozenge$) were obtained by Ca^{2+} re-addition. Data are means $\pm$ S.E.M. of five experiments.

Ca^{2+} , in that Ca^{2+} removal converted the biphasic increase in intracellular Ca^{2+} into a simple monophasic response, and subsequent replacement restored the plateau phase. It should be noted that the sustained increase in intracellular Ca^{2+} observed during the plateau phase is a dynamic balance between Ca^{2+} entry through a channel and extrusion via the Ca^{2+} -ATPase and/or $\text{Na}^{+}/\text{Ca}^{2+}$ antiport system.

The present experiments have revealed that maintenance of the plateau phase requires the continued presence of agonist, in that removal of carbachol from the receptor with the antagonist atropine removes the ability of the cell to admit Ca^{2+} (i.e. the Ca^{2+} channel closes). It is assumed that once the agonist is removed from the receptor the intracellular stores would refill in preparation for subsequent challenge. This agonist-insensitive refilling phenomenon has been observed in the parotid (Merritt & Rink, 1987a) and parietal cell (Negulescu & Machen, 1988); refilling Ca^{2+} transients could be observed in the former, but not in the latter. In the study by Negulescu & Machen (1988) the refilling process could not be detected as an increase in intracellular Ca^{2+} , indicating that Ca^{2+} must move directly from the extracellular space to the intracellular stores. More recently, in parotid acini Takemura & Putney (1989) have demonstrated a refilling transient, using fura2 (confirming the earlier work by Merritt & Rink, 1987b), indicating that, in this cell at least, the refilling route can be monitored by fura2 or that the transient could represent an overspill phenomenon. It could be argued that the present study has demonstrated a refilling transient increase in intracellular Ca^{2+} (Fig. 4), where in Ca^{2+} -free buffer discharge of the intracellular stores followed by atropine addition and Ca^{2+} repletion resulted

Fig. 7. Effect of Mn^{2+} (0.1 mM) on fura 2 fluorescence

Raw fluorescence traces are shown for excitation at 340 nm ($\square$) and 380 nm ($\blacklozenge$) (panels *a* and *b*) and at 340 nm ($\square$) and 360 nm ($\blacklozenge$) (panel *c*) in Ca^{2+} -free buffer. In panel (*a*) Mn^{2+} was added after carbachol (0.1 mM); in panels (*b*) and (*c*) Mn^{2+} was added before carbachol. Ionomycin ($2\ \mu M$) was added at the end of each trace to allow a more complete Mn^{2+} entry. Data are from a single experiment typical of two others. Data points were taken every 3.8 s.

in an increase in intracellular Ca^{2+} . However, vehicle alone resulted in the same increase, suggesting a simple re-adjustment in basal intracellular Ca^{2+} .

We have further studied the plateau phase by substituting the bivalent cation Mn^{2+} for Ca^{2+} . Fura2 has a 40-fold preference for Mn^{2+} over Ca^{2+} (Grynkiewicz *et al.*, 1985) quenching the fluorescent signal at all excitation wavelengths. Since there are no intracellular stores of Mn^{2+} , an agonist-stimulated quench is indicative of Mn^{2+} entry across the plasma membrane. Addition of carbachol in Ca^{2+} -free buffer causes, as described above, a discharge of the internal store, manifest as a spike, followed by a return to basal levels, at which time the Ca^{2+} channel is still open (shown by an increase in intracellular Ca^{2+} seen on Ca^{2+} repletion). Addition of Mn^{2+} during the plateau phase quenches the fluorescent signal at both excitation wavelengths, indicating that Mn^{2+} (or Ca^{2+}) is entering across the plasma membrane, lending further support to the concept that, as long as the receptor is occupied, the Ca^{2+} channel remains open and that if refilling of the internal store occurs via this route it must not be detected by fura2. Our data also show that Ca^{2+} -channel opening is an early event after agonist addition, since when we use the isosbestic point of fura2 (a wavelength not sensitive to changes in intracellular Ca^{2+} , but Mn^{2+} -quenchable), quenching of the fluorescent signals occurs sometime during or in conjunction with the discharge of the intracellular stores.

After the completion of this manuscript, Fisher *et al.* (1989) published data showing that in the parent SK-N-SH cell, which contains both neuronal and epithelial cell types (Ross & Biedler, 1985), oxotremorine-M induced a biphasic increase in intracellular Ca^{2+} , the initial response

being directly related to the production of total $[^3H]InsP_3$. No detailed analysis of the plateau phase was performed.

Taken collectively, the present data strongly suggest that muscarinic stimulation leads to a rapid but reversible release of Ca^{2+} from internal stores and a parallel early entry of Ca^{2+} across the plasma membrane. The difference in potency of muscarinic agonists for these components of Ca^{2+} homeostasis could suggest their independence but this has not been conclusively established here. It seems very likely that the release of intracellular stores of Ca^{2+} is mediated by $Ins(1,4,5)P_3$, as judged by the very similar time courses of production of these parameters. The Ca^{2+} channel involved in the maintained plateau-phase intracellular $[Ca^{2+}]$ seen in these cells is more complex. We have excluded the possibility of dihydropyridine Ω -conotoxin-inhibitable voltage-sensitive Ca^{2+} channels. The Ca^{2+} channel could be second-messenger operated and opened by increased $Ins(1,4,5)P_3$ or $Ins(1,3,4,5)P_4$, although this does not appear to be the case, since low ($< 0.3\ \mu M$) agonist concentrations that do not produce a significant increase in $Ins(1,4,5)P_3$ (results not shown) [or presumably in $Ins(1,3,4,5)P_4$] still produce a plateau-phase-like intracellular Ca^{2+} response (D. G. Lambert, unpublished work). Direct linkage of the muscarinic receptor to the Ca^{2+} channel via a G-protein is another possibility (Gomperts, 1983; Yatani *et al.*, 1987; Penner, 1988; Rosenthal *et al.*, 1988; Inoue & Kenimer, 1988). In a recently completed study (D. G. Lambert & S. R. Nahorski, unpublished work), preincubation of SH-SY5Y cells for 24 h with pertussis toxin (100 ng/ml) had no effect on either peak or plateau-phase intracellular Ca^{2+} or carbachol-stimulated $[^3H]$ inositol phosphate production. In parallel

experiments, pertussis toxin reversed the α_2 -adrenergic inhibition of forskolin-stimulated cyclase. These data indicate that the muscarinic receptor is not linked to the Ca^{2+} channel via a pertussis-toxin-sensitive (G_i -like) G-protein, and further may indicate that cyclic AMP is also not significant in this respect.

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