Desensitization and recovery of muscarinic and histaminergic Ca^{2+} mobilization in 1321N1 astrocytoma cells

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Intracellular free Ca^{2+} was monitored in suspensions of 1321N1 astrocytoma cells by using the Ca^{2+} indicator fura-2. The cytoplasmic Ca^{2+} concentration increased from 237 ± 6 nM to 1580 ± 170 nM within 3–5 s of addition of 300 μM -carbachol. After the peak in response, the Ca^{2+} concentration diminished, establishing a new steady state in about 1 min that was approx. 150 nM above the previous baseline. Histamine increased cytoplasmic Ca^{2+} to about 40 % of the maximal value seen with carbachol. In Ca^{2+} -free buffer each agonist elicited a normal initial increase in cytoplasmic Ca^{2+} , but the sustained portion of the response was abolished. The increase in Ca^{2+} in response to either carbachol or histamine could be completely inhibited by pretreating the cells with carbachol; the response to carbachol could be partially inhibited by pretreating the cells with histamine. The Ca^{2+} responses did not recover in the continued presence of carbachol. However, if the carbachol was washed out or if atropine was added after carbachol, the responses to agonist recovered in a time-dependent manner (half-time 3–4 min), and recovery depended on the presence of extracellular calcium. The results indicate that carbachol and histamine stimulate release of Ca^{2+} from the same intracellular Ca^{2+} store, that depletion of this store is responsible for heterologous desensitization between these two agonists, and that repletion of the agonist-sensitive Ca^{2+} pool does not occur in the continued presence of agonist or in the absence of extracellular Ca^{2+} .

INTRODUCTION

Both muscarinic and H_1 histaminergic receptors are present on 1321N1 human astrocytoma cells. Stimulation of either receptor initiates the hydrolysis of membrane phosphoinositides with the concomitant production of $\text{Ins}(1,4,5)\text{P}_3$ (Masters *et al.*, 1984; Nakahata & Harden, 1987; Ambler *et al.*, 1987). Ca^{2+} -isotope tracer studies have shown that Ca^{2+} efflux from these cells is also stimulated by both carbachol and histamine (Masters *et al.*, 1985; Nakahata *et al.*, 1986); this is consistent with the postulated role of InsP_3 as an intracellular second messenger, serving to induce the release of Ca^{2+} ions from an intracellular storage site, presumably the endoplasmic reticulum (Berridge, 1983, 1986; Williamson *et al.*, 1985).

An interesting aspect of muscarinic stimulation in these cells is that the Ca^{2+} -efflux response is rapidly desensitized in the continued presence of agonist (Masters *et al.*, 1985). The mechanism of the desensitization cannot be due to receptor down-regulation, since down-regulation of muscarinic receptors in this system requires a much longer agonist incubation period. Furthermore, the agonist-induced increase in InsP_3 is sustained in the continued presence of agonist; this suggests that the Ca^{2+} -efflux response is desensitized despite increased cellular InsP_3 . The desensitization of the Ca^{2+} response induced by agonist treatment is heterologous; for example, pretreatment of the cells with carbachol also inhibits the subsequent Ca^{2+} -efflux response to histamine (Masters *et al.*, 1985). There is also heterologous desensitization of a more distal Ca^{2+} -dependent response

in these cells, the activation of cyclic AMP phosphodiesterase (Harden *et al.*, 1986).

The purpose of the present study was to define further the mechanism of desensitization of the Ca^{2+} responses of astrocytoma cells, by using the fluorescent Ca^{2+} indicator fura-2 to monitor directly changes in cytoplasmic Ca^{2+} . The results suggest that carbachol and histamine release Ca^{2+} from the same intracellular store in astrocytoma cells. Depletion of this store occurs rapidly, and it is repleted only if agonist is removed and extracellular Ca^{2+} is present. The depletion of a common agonist-sensitive Ca^{2+} pool appears to be the mechanism of heterologous desensitization between carbachol and histamine.

METHODS

Astrocytoma cells were cultured as described previously on 100 mm-diam. culture plates (Masters *et al.*, 1984). They were utilized for fluorescence studies on day 7 of subculture. Cell suspensions were prepared by incubating the plates with 0.25 % trypsin (in Hanks solution without Ca^{2+} and Mg^{2+} ; Irvine Scientific) for 3 min at 35 °C. The trypsin was then removed by aspiration and replaced with a buffer containing 118 mM-NaCl, 5 mM-KCl, 1.2 mM- MgSO_4 , 1.2 mM- KH_2PO_4 , 1.8 mM- CaCl_2 , 10 mM-glucose, and 20 mM-Hepes. After the trypsin treatment, the cells were dislodged by repeatedly pipetting the buffer against the bottom of each plate. Once removed, the suspension was subjected to a few additional triturations to break up cell clumps.

Abbreviations used: InsP_3 , inositol trisphosphate (collectively); $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate; $\text{Ins}(1,3,4)\text{P}_3$, inositol 1,3,4-trisphosphate; $\text{Ins}(1,3,4,5)\text{P}_4$, inositol tetrakisphosphate.

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Intracellular Ca^{2+} concentrations were determined by use of the fluorescent Ca^{2+} indicator fura-2. Cells were loaded with this dye by incubating the cell suspensions at 1×10^6 – 1.5×10^6 cells/ml with $1 \mu\text{M}$ -fura-2AM, the ester form of fura-2, for 15–20 min at 37°C . Buffer of the same composition as described above was used for loading the cells with fura-2 and for the subsequent fluorescence studies. After the loading, the cells were centrifuged (2 min at 180 g) and resuspended in buffer that was free of fura-2. Fluorescence measurements were made on 2 ml samples continuously stirred in polystyrene cuvettes (Fisher; rated for use at 340 nm) and thermostatically maintained at 37°C . Fluorescence was monitored with a SPEX Fluorolog spectrofluorimeter, with excitation and emission set to 340 and 510 nm respectively. Band-passes were 4.6 nm for both the excitation and the emission wavelengths. Since the spectrofluorimeter was not equipped for excitation at dual wavelengths, the technique used was similar to that which is normally used with quin-2 (Tsien *et al.*, 1982). After monitoring of the fluorescence of the fura-2-loaded cells, each sample was sonicated (Kontes micro-ultrasonic cell disrupter) to release all of the cellular fura-2 into the buffer; since the buffer contained 1.8 mM - Ca^{2+} , this saturated the fura-2 with Ca^{2+} , resulting in the maximal fluorescence (F_{max}). EGTA (20 mM) was then added ($\text{pH} > 8.0$) to chelate the Ca^{2+} and minimize the fluorescence (F_{min}). Intracellular Ca^{2+} concentrations were calculated from the equation:

$$[\text{Ca}^{2+}]_i = \frac{F - F_{\text{min}}}{F_{\text{max}} - F} \times K_d$$

where K_d is the affinity of fura-2 for Ca^{2+} (225 nM at 37°C ; Grynkiewicz *et al.*, 1985). The fluorescence of cells incubated with fura-2AM was 8–10-fold above the autofluorescence, and the autofluorescence was not changed significantly by the drug additions or the lysis procedure.

Leakage of fura-2 molecules from the cytoplasm of loaded cells into the buffer is a common problem with the quin-2 and fura-2 techniques (Murphy *et al.*, 1986; Wollheim & Biden, 1986), and also occurred in our samples. To minimize this, each sample was loaded separately with fura-2, then centrifuged and resuspended in fura-2-free buffer just before the start of the fluorescence measurement. In some representative experiments, $50 \mu\text{M}$ - MnCl_2 was used to correct further the measurements for the residual extracellular fura-2 by the method of Murphy *et al.* (1986). These runs confirmed that the correction is relatively minor, with the Ca^{2+} concentrations and responses reported here being at most 20–30% too high.

To eliminate bias resulting from variability of the Ca^{2+} responses between cell preparations, all quantitative comparisons were performed with cells from the same batch on the same day.

The data are expressed as means $\pm$ S.E.M. unless otherwise stated.

RESULTS

Ca^{2+} responses to carbachol and histamine

The basal intracellular Ca^{2+} concentration in 1321N1 astrocytoma cells averaged $237 \pm 6 \text{ nM}$ (means $\pm$ S.E.M., $n = 75$). Addition of $300 \mu\text{M}$ -carbachol to the cells

Fig. 1. Effects of carbachol and histamine on intracellular Ca^{2+} concentration in 1321N1 astrocytoma cells

Fluorescence traces were converted into intracellular Ca^{2+} concentrations on a point-by-point basis via the equation shown in the Methods section. (a) Ca^{2+} responses to carbachol (continuous line) and histamine (broken line) in normal buffer. The two traces were arbitrarily off-set in time for clarity. (b) Ca^{2+} responses to carbachol and histamine in low- Ca^{2+} buffer (6 mM -EGTA added to the normal buffer, decreasing $[\text{Ca}^{2+}]$ from 1.8 mM to 130 nM as determined with fura-2). Although the representative responses shown in (b) are smaller than those in (a), the average initial response to either agonist in the normal- and low- Ca^{2+} buffers was the same when measured in the same cell preparation. Drug additions at the arrows were: C, $300 \mu\text{M}$ -carbachol; H, $100 \mu\text{M}$ -histamine; A, $0.6 \mu\text{M}$ -atropine; D, $10 \mu\text{M}$ -diphenhydramine.

resulted in a rapid increase in intracellular Ca^{2+} concentration, with peak values obtained 3–5 s after drug addition (Fig. 1a). The peak response to $300 \mu\text{M}$ -carbachol (increase above basal Ca^{2+}) averaged $1580 \pm 170 \text{ nM}$ (mean $\pm$ S.E.M. for ten cell preparations). The carbachol response was dose-dependent, with an EC_{50} (concn. giving 50% of maximal effect) of $\sim 30 \mu\text{M}$ (Fig. 2), and could be blocked with $0.6 \mu\text{M}$ -atropine (results not shown), which indicates that it was mediated through muscarinic receptors.

After the initial increase in Ca^{2+} induced by carbachol, the Ca^{2+} concentration diminished; by 1 min after drug addition, a new steady state was achieved. This was about 150 nM above the original basal Ca^{2+} concentration, and was maintained throughout the continued exposure to carbachol. If atropine was added to the

Fig. 2. Dose-response relationship for carbachol-stimulated increases in intracellular Ca^{2+}

Ca^{2+} response is defined as the peak intracellular Ca^{2+} concentration reached minus the basal Ca^{2+} concentration measured just before drug addition (~ 200 nm for these experiments). The peak responses to low concentrations of carbachol developed more slowly than the response to maximally effective concentrations (for $10 \mu\text{M}$ -carbachol, the response maximized in about 25 s). Symbols without error bars are for single points; for those with error bars, $n = 4$.

sample during this sustained portion of the carbachol response, the cytoplasmic Ca^{2+} concentration fell back to the baseline concentration within 10–20 s (Fig. 1a).

The Ca^{2+} response to histamine was similar to, but smaller than, the response to carbachol (Fig. 1a). The maximal response was obtained with $100 \mu\text{M}$ -histamine; doubling the histamine concentration to $200 \mu\text{M}$ did not significantly increase the response. The average response to $100 \mu\text{M}$ -histamine was 706 ± 100 nm for 12 cell preparations. The response was apparently mediated via H_1 histaminergic receptors, since it could be blocked with $1 \mu\text{M}$ -diphenhydramine (results not shown). As with carbachol, there was a sustained increase in cytoplasmic Ca^{2+} , averaging approx. 70 nm, in the continued presence of histamine. Addition of diphenhydramine during the sustained portion of the response caused the cytoplasmic Ca^{2+} concentration to return to baseline (Fig. 1a).

The dependence of the Ca^{2+} responses to carbachol and histamine on extracellular Ca^{2+} was tested by comparing the responses in normal (1.8 mM)- and low (130 nM)- Ca^{2+} buffers (Fig. 1b). In the low- Ca^{2+} buffer, the initial increase in cytoplasmic Ca^{2+} occurred normally, which is consistent with the hypothesis that these agonists mobilize Ca^{2+} from intracellular Ca^{2+} stores. In the low- Ca^{2+} buffer, however, the cytoplasmic Ca^{2+} concentration returned to the baseline value within 1 min, indicating a lack of sustained response. In the absence of a sustained response to carbachol, addition of atropine did not further decrease cytoplasmic Ca^{2+} (Fig. 1b).

To determine if carbachol and histamine were acting through the same or different mechanisms to increase Ca^{2+} , we determined if the maximal increases in cytoplasmic Ca^{2+} by these agents were additive. When $300 \mu\text{M}$ -carbachol and $100 \mu\text{M}$ -histamine were added to the cells simultaneously, the increase in cytoplasmic Ca^{2+}

Table 1. Lack of additivity between the carbachol and histamine responses

The carbachol and histamine concentrations were maximal ($300 \mu\text{M}$ and $100 \mu\text{M}$ respectively); $n = 4$.

Agent	Ca^{2+} response (nm)	
	Observed	Expected if additive
Carbachol	931 ± 177	–
Histamine	387 ± 31	–
Carbachol + histamine	975 ± 56	1320

was not different from that obtained with $300 \mu\text{M}$ -carbachol alone, and was less than what would be expected from the sum of the responses to carbachol and histamine (Table 1). Thus the responses were not additive, which suggests that the two agents increase cytoplasmic Ca^{2+} via a common mechanism.

Desensitization of the Ca^{2+} responses

If the two agonists initiate release of Ca^{2+} from a common, limited, intracellular Ca^{2+} pool, then pre-treating the cells with carbachol might deplete the pool and prevent the Ca^{2+} response to subsequent histamine addition. Indeed, when a maximally effective carbachol concentration was used for the pretreatment, subsequent addition of histamine elicited little or no increase in cytoplasmic Ca^{2+} concentration (Fig. 3a). Waiting up to 10 min between agonist additions did not promote recovery of the response to histamine. If lower doses of carbachol were used, however, resulting in a smaller initial mobilization of Ca^{2+} , the histamine response was less inhibited (results not shown).

Histamine pretreatment was also found to desensitize subsequent responses to carbachol. Table 2 gives the increase in Ca^{2+} elicited by a single challenge with a maximally effective concentration of either carbachol (column A) or histamine (column B). In each of the four experiments, the response to histamine was 40–52 % of the response to carbachol. Table 2 also shows the responses to the same maximal concentration of carbachol added to the cells 3 min after the addition of histamine (column C). After pretreatment with histamine, the carbachol response was inhibited to a degree closely corresponding to the initial response to histamine. It therefore appears that there is a carbachol-releasable pool of intracellular Ca^{2+} that is also mobilized to a lesser extent by histamine. If the cells are exposed to histamine, approx. 44 % of this pool is released; if carbachol is added after histamine, the remaining Ca^{2+} (57 % of the initial pool) can be mobilized.

Since carbachol pretreatment desensitizes the response to histamine, and vice versa, carbachol pretreatments should also desensitize subsequent responses to carbachol. To test this, fura-2-loaded cells were treated with $300 \mu\text{M}$ carbachol for 2 min and then washed by centrifugation and resuspended in carbachol-free buffer. When the cells were re-challenged with carbachol, the Ca^{2+} response in the pretreated cells averaged just 40 % of the normal carbachol response, indicating that desensitization had occurred (Fig. 4). However, since

Fig. 3. Densitization of the histamine response after carbachol pretreatment

(a) Addition of 300 μ M-carbachol (C) followed by 100 μ M-histamine (H). (b) Addition of carbachol followed by 0.6 μ M-atropine (A) and then histamine.

there was only 60% inhibition, desensitization appeared to be incomplete. A likely explanation for this is that there was partial recovery of the Ca^{2+} stores during the washing procedure, which required 2.5 min to perform. On the supposition that such recovery would require extracellular Ca^{2+} , we repeated the experiment except that the cells were washed and resuspended in buffer containing no added Ca^{2+} and 0.5 mM-EGTA (the free Ca^{2+} concentration of this buffer was less than 20 nM, as determined with fura-2). Under these conditions the carbachol pretreatment completely eliminated the response to a second challenge with carbachol (Fig. 4, — line). 'Naive' cells washed in the low- Ca^{2+} buffer responded normally to carbachol (results not shown), indicating that the brief low- Ca^{2+} treatment did not, by itself, deplete the agonist-releasable Ca^{2+} store. These results indicate that carbachol pretreatment can induce complete desensitization and that the recovery of response after the agonist is removed depends on extracellular Ca^{2+} .

Recovery of the Ca^{2+} responses

We then carried out experiments to determine how long it would take for the Ca^{2+} responses to recover after desensitizing carbachol pretreatment. To study recovery of the histamine response, we used a protocol in which carbachol (to induce desensitization), atropine (to

Table 2. Heterologous desensitization between histamine and carbachol

The values shown are the increase in Ca^{2+} (nM) above basal in response to carbachol (A) for 'naive' cells, in response to histamine (B) for 'naive' cells, and in response to carbachol for cells that were exposed to histamine for 3 min (C). Each row represents a different experiment. The values given are the mean responses for three to five determinations. The percentage values are the responses normalized to the responses elicited by 300 μ M-carbachol in 'naive' cells (column A).

Expt.	300 μ M-Carbachol (A)	100 μ M-Histamine (B)	Carbachol after histamine (C)
I	1407	568 (40 %)	818 (58 %)
II	930	386 (41 %)	504 (54 %)
III	1802	761 (42 %)	1316 (73 %)
IV	2345	1222 (52 %)	1017 (43 %)
Means...		(44 $\pm$ 3 %)	(57 $\pm$ 7 %)

Fig. 4. Desensitization of the carbachol response after carbachol pretreatment

—, Response to 300 μ M-carbachol in 'naive' cells, average of five runs; ---, carbachol response in cells pretreated with 300 μ M-carbachol for 2 min, washed, and then re-challenged with carbachol, average of five runs;, carbachol response in cells pretreated with carbachol, washed in Ca^{2+} -free buffer and re-challenged with carbachol, average of three runs.

terminate muscarinic stimulation) and then histamine were sequentially added to the cells. Under these conditions, the response to histamine recovered as a function of time after the atropine addition (Fig. 3b). Recovery of the histamine response was complete within 7 min after the atropine addition (Fig. 5a). To study recovery of the carbachol response, we used a protocol in which the cells were exposed to carbachol, washed, and then re-challenged with carbachol. Extending the period between the wash and the second carbachol addition to 7 min allowed full recovery of the response (Fig. 5b). The similarity in the recovery rate of the carbachol and histamine responses after carbachol pretreatment is further illustrated in Fig. 5(c), where the recovery for

Fig. 5. Recovery of the agonist-stimulated Ca^{2+} response in cells desensitized by carbachol pretreatment

(a) Response to histamine in either 'naive' cells ($\square$) or cells pretreated with carbachol and then atropine to allow recovery of the histamine response (▨). Recovery time is defined as the time between the atropine addition and the histamine addition. (b) Response to carbachol in either 'naive' cells ($\square$) or cells pretreated with carbachol, then washed to remove carbachol before the second carbachol challenge (▨). Recovery time is defined as the time when the cells were in agonist-free buffer between the carbachol pretreatment and re-challenge. (c) Time course of the recovery of the agonist-sensitive Ca^{2+} pool. The squares are derived from the data in (a), normalized to the carbachol response in 'naive' cells. The circles are derived from the data in (b), normalized to the histamine response in 'naive' cells. Carbachol, histamine and atropine concentrations were $300 \mu\text{M}$, $100 \mu\text{M}$ and $0.6 \mu\text{M}$ respectively. For each bar, the number of determinations is given in parentheses.

each agonist is plotted as a function of time after termination of carbachol action (by wash-out or atropine addition). The half-time for recovery of agonist response after cessation of muscarinic stimulation was 3–4 min. In both cases, the response to agonist did not recover in the absence of extracellular Ca^{2+} .

DISCUSSION

In the present study, the fluorescent probe fura-2 was used to monitor changes in the cytoplasmic Ca^{2+} concentration in astrocytoma cells subjected to muscarinic or histaminergic stimulation. The dominant feature of the fura-2 data is the large and nearly instantaneous increase in intracellular Ca^{2+} concentration induced by either carbachol or histamine. This initial Ca^{2+} response was independent of the extracellular Ca^{2+} concentration, supporting the idea that receptor activation leads to mobilization of Ca^{2+} from an intracellular store. The elevation of cytoplasmic Ca^{2+} concentration would be expected to increase Ca^{2+} extrusion from the cell via both the plasma-membrane Ca^{2+} pump (Ca^{2+} ATPase) and the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (Carafoli, 1984). This probably accounts for the large agonist-induced $^{45}\text{Ca}^{2+}$ -efflux response observed in these cells (Masters *et al.*, 1984, 1985; Nakahata *et al.*, 1986) and the rapid return of the cytoplasmic Ca^{2+} concentration to near baseline after its peak value. The agonist-induced increases in cytoplasmic Ca^{2+} concentration shown here and the Ca^{2+} efflux described previously exhibit similar dose-dependencies (Masters *et al.*, 1984; Nakahata *et al.*, 1986).

Regarding the potential second messenger linking receptor activation and Ca^{2+} release, $\text{Ins}(1,4,5)\text{P}_3$ has been shown to release Ca^{2+} from an intracellular store in a variety of permeabilized cell preparations (reviewed by Berridge, 1986). In 1321N1 cells, $\text{Ins}(1,4,5)\text{P}_3$ is increased within 25–30 s of carbachol addition, which is slower than the Ca^{2+} response (Ambler *et al.*, 1987). There is, however, rapid conversion of the isomers of InsP_3 in this cell line, which make quantitative comparisons between the $\text{Ins}(1,4,5)\text{P}_3$ and Ca^{2+} responses difficult. For example, $\text{Ins}(1,3,4)\text{P}_3$ is produced within 6 s of agonist addition; since $\text{Ins}(1,3,4)\text{P}_3$ is apparently derived from $\text{Ins}(1,4,5)\text{P}_3$ via the intermediate $\text{Ins}(1,3,4,5)\text{P}_4$ (Irvine *et al.*, 1986; Ambler *et al.*, 1987), the data suggest that $\text{Ins}(1,4,5)\text{P}_3$ production actually begins soon after addition of agonist. It is conceivable that the $\text{Ins}(1,4,5)\text{P}_3$ initially generated mobilizes Ca^{2+} before being phosphorylated to $\text{Ins}(1,3,4,5)\text{P}_4$.

We previously suggested a relationship between InsP_3 formation and Ca^{2+} mobilization in these cells, based on comparison of the InsP_3 and Ca^{2+} -efflux responses to a variety of agonists (Masters *et al.*, 1984; Evans *et al.*, 1985). More direct evidence for such a relationship was that phorbol myristate acetate, which blocks muscarinic-receptor-stimulated InsP_3 formation, also prevents the stimulation of $^{45}\text{Ca}^{2+}$ efflux (Orellana *et al.*, 1985). For InsP_3 production, carbachol is more efficacious than histamine (Nakahata *et al.*, 1986; Nakahata & Harden, 1987), which correlates with the differential ability of these agents to mobilize Ca^{2+} .

A difference between the Ca^{2+} response and the phosphoinositide response is that Ca^{2+} mobilization and efflux rapidly return to near-normal values, whereas hydrolysis of membrane phosphoinositides continues as

long as carbachol is present (Masters *et al.*, 1985). One possibility is that, although the inositol phosphates remain increased in the presence of agonist, $\text{Ins}(1,4,5)\text{P}_3$ (the isomer of InsP_3 shown to mobilize Ca^{2+} ; Berridge, 1986) may decrease, owing to conversion into $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(1,3,4)\text{P}_3$ (Irvine *et al.*, 1986). However, $\text{Ins}(1,4,5)\text{P}_3$ remains substantially increased during continued exposure to carbachol (Ambler *et al.*, 1987; Nakahata & Harden, 1987). Thus the decline in the Ca^{2+} response does not result from a decrease in the Ca^{2+} -releasing second messenger; instead, it appears more likely that the agonist-releasable Ca^{2+} pool is depleted.

In the continued presence of either agonist, a new steady state was established, in which the cytoplasmic Ca^{2+} concentration was slightly above the basal value. The sustained increase in cytoplasmic Ca^{2+} differed from the initial response to agonist in that it depended on the extracellular Ca^{2+} concentration. Thus it may correspond to the small $^{45}\text{Ca}^{2+}$ influx response observed previously (Masters *et al.*, 1984). More recently, both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ have been proposed to play a second-messenger role in agonist-induced Ca^{2+} influx (Kuno & Gardner, 1987; Irvine & Moor, 1986). Both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ are produced in the astrocytoma cells (Ambler *et al.*, 1987), but further studies are needed to determine if either of these agents mediates Ca^{2+} influx.

The Ca^{2+} responses to carbachol and histamine in 1321N1 cells were not additive. This suggests that these agonists induce the release of Ca^{2+} from the same intracellular pool. Further evidence for this was the demonstration of heterologous desensitization between these agonists. Pretreating the cells with maximal carbachol concentrations nearly abolished subsequent Ca^{2+} responses to histamine, as if the carbachol pretreatment had fully depleted the common releasable Ca^{2+} store. Histamine pretreatments also inhibited subsequent challenges by carbachol; the inhibitory effect was not as strong as in the converse case, but this is consistent with the lower efficacy of histamine compared with carbachol in inducing the initial Ca^{2+} release. Lesser inhibitory effects were also obtained with carbachol if sub-maximal concentrations were used for the desensitizing pretreatment.

The portion of the pool sensitive to carbachol and that sensitive to histamine both refill at the same rate, which is further evidence that it is a shared Ca^{2+} pool. The recovery is not seen if extracellular Ca^{2+} is omitted, suggesting that recovery results from refilling of the hormone-sensitive Ca^{2+} pool with Ca^{2+} from outside the cell. In the absence of extracellular Ca^{2+} , the agonist-releasable pool does not refill with Ca^{2+} from other intracellular Ca^{2+} -containing organelles such as the mitochondria. The pool also refills in the absence of a general elevation in cytoplasmic Ca^{2+} concentration; this is consistent with the model proposed by Putney (1986) for replenishment of agonist-sensitive Ca^{2+} stores.

The results with astrocytes resemble those obtained with a number of other systems, in which quin-2 or fura-2 have been used to monitor agonist-induced Ca^{2+} responses, including smooth-muscle cells (Matsumoto *et al.*, 1986), gastric-gland cells (Chew, 1986), PC12 cells (Pozzan *et al.*, 1986), parotid cells (Takemura, 1985), pancreatic acinar cells (Merritt & Rubin, 1985), hepatocytes (Joseph *et al.*, 1985) and NG108-15 neuroblastoma ×

glioma hybrids (Osugi *et al.*, 1986). In each case, stimulation of the cells by agonist leads to an initial transient increase in cytoplasmic Ca^{2+} owing to release of intracellular Ca^{2+} stores followed by a sustained response that depends on extracellular Ca^{2+} ; this may be a general response pattern to agonists working through the phosphoinositide cycle (Rasmussen & Barrett, 1984).

Desensitization between different agonists has been observed for Ca^{2+} -dependent responses such as K^{+} -permeability changes in parotid glands (Putney, 1977), contraction in smooth muscle (Siegel *et al.*, 1984), phosphorylase activation in hepatocytes (Breant *et al.*, 1981; Kirk & Creba, 1982), and activation of cyclic AMP phosphodiesterase in 1321N1 astrocytoma cells (Harden *et al.*, 1986). Confirmation that this desensitization is at the level of agonist-induced Ca^{2+} mobilization has been obtained with $^{45}\text{Ca}^{2+}$ in pancreatic acinar cells (Stolze & Schulz, 1980), and with quin-2 in gastric-gland cells (Chew, 1986) and hepatocytes (Joseph *et al.*, 1985). The present study is consistent with these previous reports, and additionally defines the time course of recovery of the Ca^{2+} response after desensitization in 1321N1 cells.

In summary, muscarinic- and histaminergic-receptor stimulation in 1321N1 astrocytoma cells induces the hydrolysis of membrane phosphoinositides to form InsP_3 , and leads to rapid elevation of the cytoplasmic free Ca^{2+} concentration. The initial Ca^{2+} increase appears to be entirely accountable by the agonist-stimulated release of Ca^{2+} from an intracellular Ca^{2+} store. In the continued presence of agonist, the cytoplasmic Ca^{2+} concentration returns to near basal; the cytoplasmic store, however, does not refill as long as InsP_3 remains increased, and replenishment depends on extracellular Ca^{2+} . Heterologous desensitization between carbachol and histamine appears to result from depletion of the common Ca^{2+} pool.

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