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Carbachol induces secretion in a mast cell line (RBL-2H3) transfected with the m1 muscarinic receptor gene

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The possibility that the m1 muscarinic receptor subtype can induce release of intracellular granules and transmitters was studied by transfecting a cultured mast cell line, RBL-2H3 cells, with the m1 receptor gene. Comparisons were made between carbachol- and antigen-induced activation of various secretory responses. Like antigen, carbachol stimulated inositol phospholipid hydrolysis and release of arachidonic acid with concomitant dose-dependent secretion of granular contents. Carbachol also stimulated a biphasic increase in intracellular calcium, as measured by single cell fura-2 measurements. Although the kinetics of the carbachol-induced rise in intracellular calcium differed from that induced by antigen, they both utilized the same intracellular pool of calcium, and the second phase of the rise in intracellular calcium was dependent on extracellular calcium in both cases. Thus, the m1 muscarinic receptor activates release of granules by a mechanism ostensibly similar to that of antigen.

Cloned m1 muscarinic receptor; Expression (RBL-2H3 cell); Intracellular signal; Exocytosis

1. INTRODUCTION

Muscarinic receptors are widely distributed throughout the central nervous system and periphery, where they are thought to modulate secretion and neurotransmitter release [1,2]. Recently 5 subtypes of muscarinic receptor (m1–m5) were identified using molecular genetic techniques [3,4]. Little is known of the identity of the muscarinic receptor subtypes involved in secretion and neurotransmitter release and even less is known of the mechanisms by which muscarinic receptors control these processes. This study attempts to address these questions using a cultured rat mast cell line, RBL-2H3 cells, transfected with the m1 muscarinic receptor. RBL-2H3 cells are widely used as an experimental model for study of the secretory process in mast cells. Much of what is known of the mechanisms leading to secretion in these cells has been determined by use of antigen-stimulated aggregation of IgE receptors on the cell surface. These cells secrete granules that contain an array of readily quantifiable substances such as histamine, 5-hydroxytryptamine and hexosaminidase. The intervening signals thought to promote the secretory response include hydrolysis of inositol phospholipids and phosphatidylcholine, the mobilization of intracellular and extracellular calcium and the activation of protein kinases [5]. The m1 muscarinic receptor has been shown to stimulate phosphatidylinositol metabolism in a variety of cells [3,4] and to induce release of intracellular calcium [6,7]. Therefore, it is thought to be a likely candidate for stimulating release of intracellular granules and the RBL-2H3 cells would be an appropriate cell to test this possibility. Here we compare the effects of carbachol with those of antigen with respect to these responses in transfected RBL-2H3 cells.

Abbreviations: $[Ca^{2+}]_i$, concentration of free cytosolic Ca^{2+}; IgE, immunoglobulin E; EGTA, ethylene glycol-bis-(β aminoethylether) *N,N,N'N'*-tetraacetic acid; DNP-BSA, dinitrophenylated bovine serum albumin.

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2. MATERIALS AND METHODS

2.1. *Materials*

DNP-specific IgE and the antigen, $DNP_{25}BSA$ were gifts from Dr Henry Metzger (NIAMS, NIH). Carbachol was purchased from Aldrich, Milwaukee and atropine from Fisher Scientific Co., New York. *p*-Nitrophenyl-*N*-acetyl-β-D-glucosamide was obtained from Sigma, St. Louis, MO.

2.2. *Cell transfection*

RBL-2H3 cells were transformed by viral infection with the expression vector pDs containing both the human m1 muscarinic receptor subtype gene and the neomycin resistance gene [8]. Cells were selected with G418 for 2 weeks and subsequently subcloned. Binding studies using ^{3}[H]*N*-methylscopolamine [9] revealed subclones expressing various receptor levels. The subclone used here expressed muscarinic

receptor levels of 481 fmol/mg protein. Prior to transfection, RBL-2H3 cells displayed no muscarinic receptor binding nor a response to carbachol [10].

2.3. *Cell Culture*

The transfected cell line was maintained in culture and plated in 24-well plates by use of previously described techniques [11]. Experimental techniques and reagents were as reported elsewhere [11,12,13]. Experiments were performed in a glucose–saline, PIPES (1,4-piperazinediethane sulfonic acid) buffered medium that contained 1 mM Ca^{2+}. For the Ca^{2+}-free medium, the Ca^{2+} was omitted and replaced with 0.1 mM EGTA [12].

2.4. *Measurement of hexosaminidase*

Secretion of hexosaminidase into medium was determined as follows: 10 μl of medium and cell lysate (in 1 ml 0.1% Triton X-100) were incubated with 10 μl of 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide in 0.1 M sodium citrate buffer (pH 4.5) at 37°C for 1 h. At the end of the incubation, 250 μl of a 0.1 M Na_2CO_3/$NaHCO_3$ buffer (pH 4.5) was added. Absorbance was read at 400 nm. Values (mean ± SEM) were expressed as the actual release (percent of total hexosaminidase) after correction for spontaneous release (2–3%) or as a percent of maximal response. [^{3}H]5-Hydroxytryptamine [13] and hexosaminidase were released at identical rates (data not shown).

2.5. *Measurement of phospholipid hydrolysis*

Phospholipids were labeled by overnight incubation of cultures with [^{3}H]inositol and [^{14}C]arachidonic acid [13]. Generation of [^{3}H]inositol phosphates [11,12] and release of [^{14}C]arachidonic acid into the medium [13] were calculated as a percent of either ^{3}H- or ^{14}C-radiolabeled phospholipid that was present in unstimulated cultures. Otherwise values (mean±SEM) were expressed as a percent of maximal response.

2.6. *Measurement of* $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was determined in suspensions of quin2-loaded cells [11,12]. For measurement of $[Ca^{2+}]_i$ in individual cells, however, cells were grown on quartz cover slips and loaded with fura-2 acetoxymethylester (Molecular Probes, OR) (1 μM for 60 min). The cover slip was placed in a Dvorak-Stottler Chamber and maintained at 37°C. A plastic spacer was placed in the chamber to minimize dead space (50 μl). Reagents were dissolved in the Ca^{2+}-containing or Ca^{2+}-free glucose–saline buffered medium [12]. Fura-2 fluorescence was measured at 510 nm (excitation at 340 and 380 nm) and fluorescence was expressed as a ratio of fluorescence (R) at the 2 excitation wavelengths. The calculated $[Ca^{2+}]_i$ was determined as described previously [14]. As will be described elsewhere, the values for K_d of fura-2 (230 nM), R_{max} and R_{min} were determined in thin films containing buffers of different concentrations of Ca^{2+} exactly as described [15]. Fluorescence was determined in a Deltascan fluorimeter with photon detectors (Photon Technology International Inc. NJ).

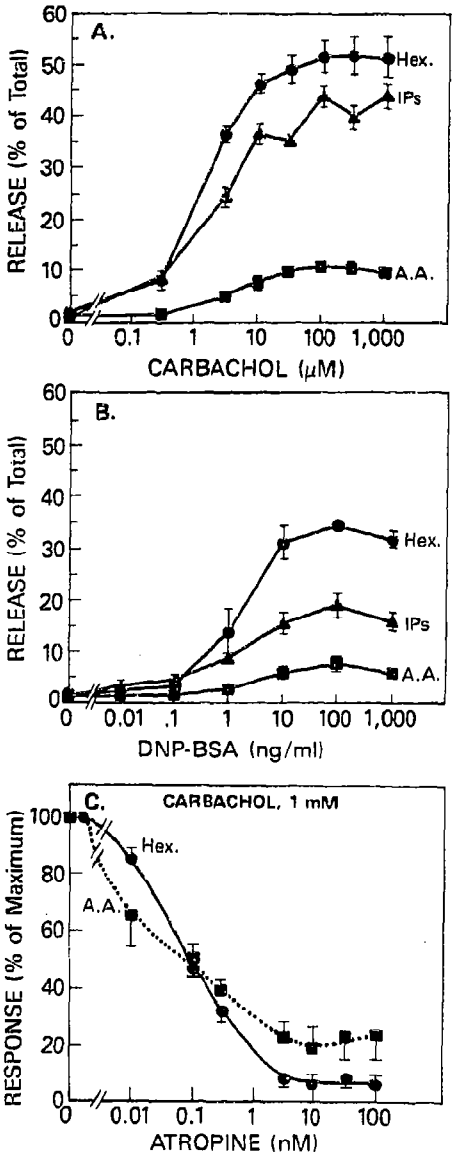

Fig. 1. Release of arachidonic acid (A.A.), inositol phosphates (IPs) and hexosaminidase (Hex) in response to different concentrations of carbachol (Panel A) or the antigen (DNP-BSA)(Panel B). Atropine inhibited the responses to carbachol (Panel C) but not those to antigen (not shown). Values are mean values ±SEM from 3 experiments.

3. RESULTS

Carbachol (Fig. 1A), like antigen (Fig. 1B), caused a concentration-dependent hydrolysis of inositol phospholipids, release of arachidonic acid and secretion of hexosaminidase. Half maximal responses (EC_{50}) occurred at about 3 μM carbachol (mean of 3 experiments). Maximal responses to carbachol were invariably larger than those to antigen (compare panels A and B, Fig. 1). Atropine inhibited the stimulation of cells by carbachol (Fig. 1C), but not that by antigen (data not shown).

Carbachol at concentrations >1 μM (Fig. 2A) also elicited increases in $[Ca^{2+}]_i$ in suspensions of quin2-loaded cells. $[Ca^{2+}]_i$ reached a maximum within 30 s and then rapidly declined to achieve steady-state levels of 200–400 nM (Fig. 2A) for at least 20 min (data not shown). The increase in $[Ca^{2+}]_i$ could be prevented (data not shown) or aborted (Fig. 2B) by addition of atropine which did not interfere with the antigen-stimulated increase in $[Ca^{2+}]_i$ (Fig. 2B). Both the initial increase (Fig. 2C) and the sustained phase of elevated $[Ca^{2+}]_i$ were dependent on concentration of carbachol. Only the initial transient increase was apparent, however, in the absence of external Ca^{2+} or when Ca^{2+} entry was blocked by addition of 10 μM La^{3+} (data not shown). Thus the contribution of intracellular and extracellular sour-

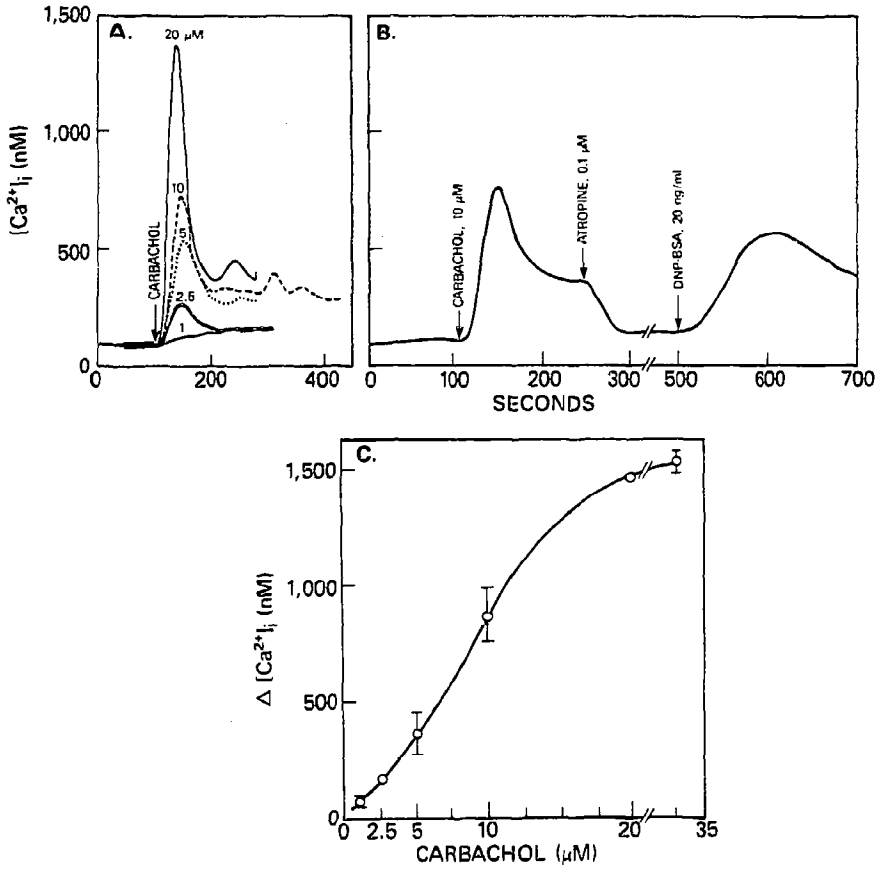

Fig. 2. Changes in $[Ca^{2+}]_i$ as measured in suspensions of quin2-loaded cells, in response to different concentrations of carbachol (Panel A) or to the sequential addition of carbachol, atropine and antigen in the same sample of cells (Panel B). Panel C shows the maximal increase in $[Ca^{2+}]_i$ (mean ±SEM from a total of 6 experiments). Points without bars were the mean of 2 observations. The maximal response ($\Delta[Ca^{2+}]_i$) to DNP–BSA in these experiments was 585±80 nM.

ces of Ca^{2+} could be clearly distinguished. This distinction was not as obvious in antigen-stimulated cells in which the maximal increases in $[Ca^{2+}]_i$ were <40 nM in the absence of Ca^{2+} or presence of La^{3+} [11,12]

Measurement of $[Ca^{2+}]_i$ with fura-2 in individual cells revealed the following. Firstly, both the initial transient and the sustained phases of the increase in $[Ca^{2+}]_i$ were still evident after carbachol stimulation. The sustained phase was apparent either as slow oscillations in $[Ca^{2+}]_i$ (Fig. 3) or a constant steady-state level reminiscent of that observed in suspensions of cells. In either case the response was of immediate onset and was rapidly reversed on removal of carbachol (Fig. 3). Secondly, antigen caused a different response. As noted by others [15], there was a delay of variable duration (up to 100 s), and then a sudden increase in $[Ca^{2+}]_i$ (Fig. 3). This was followed by a very slow (up to 60 min) decline in $[Ca^{2+}]_i$ sometimes with superimposed oscillations in $[Ca^{2+}]_i$. However, pre-depletion of intracellular stores of Ca^{2+} by stimulation of the cells with carbachol, in the absence of external Ca^{2+}, blocked further responses to either carbachol or antigen. Provision of external Ca^{2+} allowed an immediate response to antigen (Fig. 4). Conversely, pre-depletion of the stores with antigen, in the absence of external Ca^{2+}, blocked the response to carbachol (Fig. 4). Identical results were obtained whether experiments were conducted in Ca^{2+}-free medium as

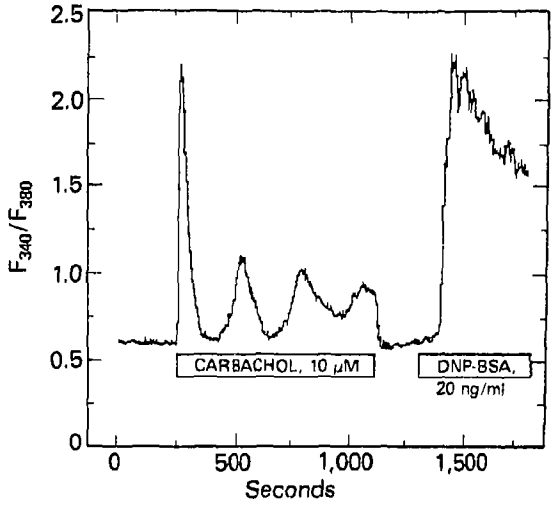

Fig. 3. Changes in $[Ca^{2+}]_i$ in a single cell (loaded with fura-2) during perfusion with 10 μM carbachol followed by antigen (20 ng/ml, DNP–BSA). The chamber was perfused with a saline-buffered medium that contained 1 mM Ca^{2+} [11].

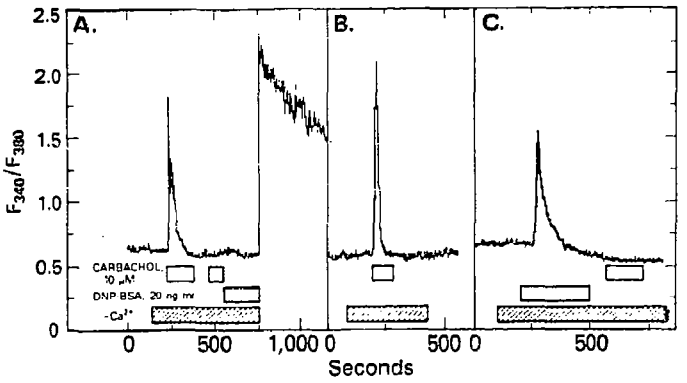

Fig. 4. Demonstration that Ca^{2+} is recruited from the same intracellular pool by carbachol and antigen. Traces from 3 representative fura-2-loaded cells are shown. Panel A: the cell was exposed twice to 10 μM carbachol (open bars) and then antigen (stippled bar) in the absence of external Ca^{2+} and presence of 0.1 mM EGTA (striped bar) before reprovision of 1 mM Ca^{2+} (no bar). The large increase in $[Ca^{2+}]_i$ at 750 s was due to residual antigen attached to cell-bound IgE. Panel B: the cell was exposed to 10 μM carbachol alone in the absence of external Ca^{2+} and EGTA (striped bar) before reprovision of Ca^{2+} (no bar) to indicate that refilling of internal stores of Ca^{2+} by itself was not associated with increases in $[Ca^{2+}]_i$. Panel C: the cell was first exposed to antigen (stippled bar) and then carbachol (open bar) in the absence of Ca^{2+}. In this situation provision of Ca^{2+} evoked an immediate response (not shown) to the antigen that remains bound to surface IgE.

described or Ca^{2+} entry was blocked by addition of 10 μM La^{3+} to a Ca^{2+}-containing medium (data not shown).

4. DISCUSSION

Activation of the m1 muscarinic receptor by carbachol resulted in the release of secretory granules from RBL-2H3 cells transformed with the m1 receptor gene. Carbachol and antigen activated the same responses in these cells, causing hydrolysis of inositol phospholipids, arachidonic acid release and release of calcium from a common pool, presumably sensitive to inositol 1,4,5-trisphosphate. Both caused a biphasic increase in intracellular calcium, the slow secondary phase of which was dependent on extracellular calcium. This is similar to that observed in glandular tissue on activation of muscarinic receptors, where a secondary slow rise in intracellular calcium and continued secretion is dependent on extracellular calcium [16]. Also both antigen [17] and m1 receptors [10] appear to activate similar calcium-dependent ionic conductances [10,17] and pathways for influx of $^{45}Ca^{2+}$ (unpublished data).

It appears that carbachol and antigen utilize similar mechanisms to activate secretion in the RBL cell. The pathways, however, must differ somewhat, as tyrosine phosphorylation of the phospholipase C γ_1 isozyme [18] is observed upon stimulation with antigen, but not with carbachol (unpublished data). Divergence from the antigen-activated secretory pathway has been observed with adenosine-mediated activation of phospholipase C which is pertussis toxin-sensitive, whilst the antigenic response is not [11]. Thus, further studies comparing the secretory responses of muscarinic receptors to those of antigenic stimulation may clarify the molecular basis of these differences.

Finally, secretion of hexosaminidase or radiolabeled 5-hydroxytryptamine can be measured with a high degree of precision in 96-well culture plates [19]. The transfected RBL-2H3 cells may, therefore, be useful for drug screening applications.

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REFERENCES

[1] Chesselet, M.-F. (1984) Neuroscience 12, 347–375.
[2] Brown, J.H. (ed.) (1989) The Muscarinic Receptors, Humana Press, Clifton, New Jersey.
[3] Hulme, E.C., Birdsall, N.J.M. and Buckley, N.J. (1990) Annu. Rev. Pharmacol. Toxicol. 30, 633–673.
[4] Jones, S.V.P. and Brann, M.R. (1991) In: Molecular Approaches to Receptors that Couple to G-Proteins. Muscarinic Acetylcholine Receptors (Brann, M.R. ed) Birkhauser, Boston (in press).
[5] Kinet, J.-P. and Metzger, H. (1990) In: Fc Receptors and the Action of Antibodies (H. Metzger ed.) American Society for Microbiology, pp. 239–259, Washington, DC.
[6] Neher, E., Marty, A., Fukuda, K., Kubo, T. and Numa, S. (1988) FEBS Letters 240, 88–94.
[7] Jones, S.V.P., Barker, J.L., Goodman, M.B. and Brann, M.R. (1990) J. Physiol. 421, 409–519.
[8] Gutkind, J.S., Novotny, E.A., Brann, M.R. and Robbins, K.C. (1991) Proc. Natl. Acad. Sci. USA (in press).
[9] Buckley, N.J., Bonner, T.I., Buckley, C.M. and Brann, M.R. (1989) Mol. Pharmacol. 35, 469–476.
[10] Jones, S.V.P. (1991) Neurosci. Soc. abstr. (in press).
[11] Ali, H., Cunha-Melo, J.R., Saul, W.F. and Beaven, M.A. (1990) J. Biol. Chem. 265, 745–753.
[12] Macyama, K., Hohman, R.J., Ali, H., Cunha-Melo, J.R., and Beaven, M.A. (1988) J. Immunol. 140, 3919–3927.
[13] Collado-Escobar, D., Ali, H. and Beaven, M.A. (1990) J. Immunol. 144, 3449–3457.
[14] Grynkiewicz, G. Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[15] Millard, P.J., Ryan, T., Webb, W.W. and Fewtrell, C. (1989) J. Biol. Chem. 264, 19730–19739.
[16] Morris, A.P., Gallacher, D.V., Irvine, R.F. and Petersen, O.H. (1987) Nature 330, 653–655.
[17] Jones, S.V.P., Cunha-Melo, J.R., Beaven, M.A. and Barker, J.L. (1987) Soc. Neurosci. abstr. 13, 106.
[18] Goldschmidt-Clermont, P.J., Kim, J.W., Machesky, L.M., Rhee, S.G. and Pollard, T.D. (1991) Science 251, 1231–1233.
[19] Baird, B., Sajewski, D. and Mazlin, S. (1983) J. Immunol. Methods 64, 365–375.