Vol. 147, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1987 Pages 182-188

CALCIUM MOBILIZATION BY MUSCARINIC RECEPTORS IN HUMAN ASTROCYTOMA CELLS: MEASUREMENTS WITH QUIN 2

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Received July 6, 1987

SUMMARY: Activation of muscarinic cholinergic receptors on 1321N1 human astrocytoma cells leads to Ca^{2+} mobilization as measured by quin 2 fluorescence. Acetylcholine and methacholine were full and potent agonists, while carbachol and muscarine, were fully efficacious but 6- and 10-fold less potent than acetylcholine. The carbachol-induced Ca^{2+} response was also observed in absence of extracellular Ca^{2+} and was blocked by muscarinic receptor antagonists but not by organic Ca^{2+} channel blockers, tetrodotoxin (TTX), tetraethylammonium (TEA) or metal cations, suggesting that Ca^{2+} is mobilized from intracellular storage sites rather than through plasma membrane ion channels. Muscarinic receptor-mediated Ca^{2+} release was also detected in kidney epithelial cells but not in rat fibroblasts, glial cells or differentiated neuroblastoma x glioma hybrid cells.

A variety of hormone receptors stimulate phosphoinositide (PI) breakdown, elevate intracellular Ca^{2+} levels $[Ca^{2+}]_i$, and alter cyclic AMP and cyclic GMP formation. Activation of muscarinic receptors on 1321N1 cells attenuates cyclic AMP production via a Ca^{2+}-dependent phosphodiesterase (1-4) and also rapidly stimulates PI breakdown leading to an increase in $[Ca^{2+}]_i$ (5). PI turnover and Ca^{2+} mobilization are both induced by muscarinic agonists with similar efficacy and potency and can be concomitantly blocked by phorbol esters which activate C-kinase, suggesting a causal relationship between these two responses (6,7). There is little information, however, on the source of Ca^{2+} by which $[Ca^{2+}]_i$ levels are increased. Changes in $[Ca^{2+}]_i$ were previously measured using ^{45}Ca efflux. In this study, we used the fluorescent Ca^{2+} indicator quin 2, to pharmacologically characterize muscarinic receptor function and to measure changes in $[Ca^{2+}]_i$ in 1321N1 cells. Our data suggest that muscarinic receptor activation leads to Ca^{2+} release from internally

ABBREVIATIONS: $[Ca^{2+}]_i$, intracellular free calcium concentration; DHP, dihydropyridine; $[Ca^{2+}]_{ex}$, extracellular calcium; HEPES, N-2-Hydroxyethyl-piperazine-N'-2-ethane sulfonic acid; QNA, quinuclidinyl atrolactate; QNX, quinuclidinyl xanthine carboxylate.

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0006-291X/87 $1.50

sequestered Ca^{2+} stores (mobilization), rather than through an influx of extracellular Ca^{2+} through voltage-operated Ca^{2+} channels (VOC). Preliminary results were presented previously (8).

Materials and Methods

Materials: Pirenzepine was a gift from Dr. Barry Wolfe, University of Pennsylvania, isomers of QNA and QNX were gifts of Dr. J. Rsezotarski, Nova Pharmaceutical Corporation, (Baltimore, MD). Ca^{2+} channel antagonists were obtained from Miles Pharmaceutical (Westhaven, CT), or Janssen Pharmaceutical (Beerse, Belgium), oxybutynin and diltiazem from Marion Laboratories (Kansas City, MO), and ionomycin from Calibiochem (San Diego, CA).
Cell Cultures: 1321N1 cells kindly provided by Dr. T. K. Harden (University of N. Carolina, Chapel Hill, NC), were cultured as previously described (1) and used between passages 2 to 30. For experiments, cultures were fed on day 6 and harvested the next day with trypsin (0.25%) for 1-2 min at 37°C. NG108-15 (neuroblastoma x glioma), and glial cells, C6BU-1, obtained from Dr. M. Nirenberg (NIH, Bethesda, MD) and kidney epithelial cells (MDCK) from Dr. M. Klagsbrun (Harvard University, Boston, MA) were cultured as previously described (9,10). NG108-15 cells were differentiated with dibutyryl cAMP (1.0 mM, 4 days).

Dispersed cells in media (5×10^6 cells/ml) were incubated with 100 μM quin 2/AM for 60 min at 37°C. Cells were then diluted in ice cold media, centrifuged and washed twice in ice cold Hepes Buffer (130 mM NaCl, 5.0 mM KCl_2, 6.0 mM glucose, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$ and 20 mM Hepes, pH 7.4).
$[Ca^{2+}]_i$ Measurements: Fluorescence was monitored with a Perkin-Elmer LS-5 Spectrophotometer at excitation and emission wavelengths of 339 nm and 492 nm, 5 and 20 nm slit width, respectively, at 37°C with or without test agents. Cells were preincubated with buffer (control) or antagonists for 1 min prior to carbachol addition. IC_{50} values defined as drug concentrations that give a half maximal fluorescence change were calculated using a Hewlett Packard IIC linear regression and standard deviation program. Data is reported as mean ± S.E.M. $[Ca^{2+}]_i$ values were calculated as described by Tsien et al., with some modification (11,12). Statistical significance was assessed by paired T-test (13).

Results

Quin 2 loaded 1321N1 cells treated with carbachol (100 μM), showed an acute increase in fluorescence (< 20 sec, Fig. 1A), reflecting an elevation of $[Ca^{2+}]_i$ (~1.5 fold). Resting $[Ca^{2+}]_i$ levels of 170 ± 10 nM were obtained similar to $[Ca^{2+}]_i$ in lymphocytes, platelets, hepatocytes, neutrophils and cultured cells (14-17). Preincubation with the muscarinic antagonist, atropine (10 μM, 1 min) eliminated the fluorescent signal (Fig. 1B). In contrast, the DHP antagonist nimodipine (1 μM), and the divalent metal cation Cd^{2+} did not inhibit the Ca^{2+} response to carbachol (Fig. 1C, Table 1). The Ca^{2+} response was also observed in presence of EGTA (2 mM)(Fig. 1D), suggesting that Ca^{2+} mobilization was independent of $[Ca^{2+}]_{ex}$. Also, depolarization with 50 mM KCl (Fig. 1E) or KCl + BAY K8644 (not shown) did not induce Ca^{2+} transients. These results suggest that Ca^{2+} release is mediated through muscarinic receptors on 1321N1 cells, presumably from internally sequestered Ca^{2+} stores rather than through DHP-sensitive Ca^{2+} channels.

Muscarinic receptor agonists mobilized Ca^{2+} in a dose-dependent manner. Acetylcholine and methacholine were full and potent agonists while carbachol and muscarine, which were equally efficacious, were 6- and 10-fold less potent than acetylcholine, respectively. Oxotremorine and arecoline, which were

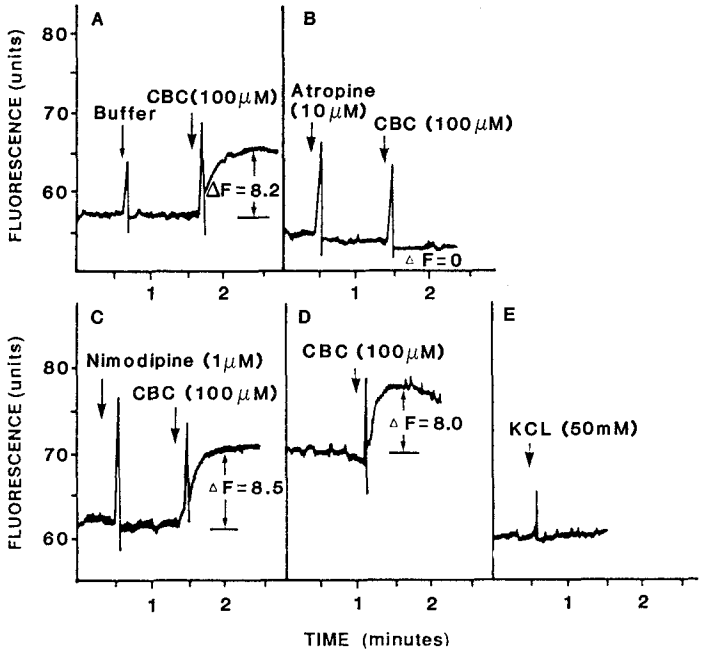

Figure 1. Typical tracings of monitoring fluorescence (F) from quin-2 loaded cells. Cells in Hepes-buffered saline were stirred continuously at 37°C with a magnetic stirrer, and treated with buffer (panel A), atropine (10 μM) (panel B), or nimodipine (1 μM) (panel C), after a steady baseline was obtained. After 1 min, 100 μM carbachol was added to the cuvettes. Parallel cultures were resuspended in buffer in 0 mM $CaCl_2$ containing 2 mM EGTA prior to carbachol addition (panel D), or treated with 50 mM KCl (panel E). Changes in fluorescence (ΔF) were calculated from the difference between final and initial (F) values. Representative traces for each protocol are shown.

partial agonists based on ^{45}Ca efflux (18) were not active here even at 100 μM (Table 1). This difference is not due to the ability of agonists to access receptors, since oxotremorine blocked $[Ca^{2+}]_i$ increases induced by carbachol (data not shown). Oxotremorine also competes with high affinity for [^{3}H] quinuclidinyl benzilate binding sites in membranes from these cells (1). Our data, however, agrees well for the potency and efficacy values of full agonists for both PI and ^{45}Ca efflux responses. Thus, carbachol and acetylcholine had EC_{50} values of about 10 μM and 1.0 μM, respectively, by both methods of Ca^{2+} determination (5, Table 1).

Several muscarinic receptor antagonists inhibited the carbachol-induced Ca^{2+} enhancement (Table 1). Scopolamine was 10- and 100-fold more potent than atropine and the "M_1-selective" antagonist, pirenzepine, respectively. Among the QNA isomers, (R,R)-QNA was the most potent, about 60 - 400 times more potent than (R,S), (S,R) and (S,S), yielding the same rank order of potency as before (19), and the QNX isomers showed a ~ 60 fold relative difference in potency. Dexetimide was more potent than levetimide by three orders of magnitude. Nicotine and tubocurarine, were both ineffective even at high concentrations. In contrast to muscarinic receptor antagonists, the DHP Ca^{2+}

Table 1

Ca^{2+} MOBILIZATION BY MUSCARINIC RECEPTOR AGONISTS AND REVERSAL BY MUSCARINIC ANTAGONISTS BUT NOT ION CHANNEL BLOCKERS

Agonists	EC_{50} ± S.E (μM)
Acetylcholine	1.1 ± 0.02
Methacholine	3.4 ± 0.03
Carbachol	6.2 ± 0.03
Muscarine	9.9 ± 0.05
Arecoline (0.1 mM)	NA
Oxotremorine (0.1 mM)	NA
Nicotine (0.1 mM)	NA
Antagonists	**IC_{50} ± S.E. (μM)**
Scopolamine	3 ± 1
Atropine	25 ± 2
Oxybutynin	34 ± 3
Pirenzepine	491 ± 7
(R,R)-QNA	6 ± 2
(R,S)-QNA	373 ± 7
(S,R)-QNA	2152 ± 37
(S,S)-QNA	2676 ± 14
(R)-QNX	4 ± 1
(S)-QNX	238 ± 15
Dexetimide	8 ± 1
Levetimide	8714 ± 37
Tubocurarine (1.0 mM)	NA
Nifedipine (10 μM)	NA
Cinnarizine (10 μM)	NA
Verapamil (1 μM)	NA
Diltiazem (10 μM)	NA
TTX (3 μM)	NA
TEA (10 μM)	NA
Cadmium (50 μM)	NA
Lanthanum (100 μM)	NA

NA = Not active at indicated concentrations.

Quin 2 loaded 1321N1 cells were treated with various concentrations of drugs. Antagonists were added 1 min prior to carbachol (100 μM). EC_{50}/IC_{50} values represent drug concentrations that produced a half maximal response and were calculated from dose response curves by linear regression. Values shown are means ± S.E. of 3 - 5 experiments performed on different batches of cells.

channel antagonist, nifedipine, as well as other classes of Ca^{2+} channel antagonists, which are potent inhibitors (nM range) of voltage operated Ca^{2+} channels (VOC) (14,20), and TTX and TEA, blockers of Na^+ and K^+ channels respectively, did not inhibit carbachol-induced Ca^{2+} release (Table 1). The metal cations Cd^{2+} and La^{3+}, which also inhibit VOC, failed to block $[Ca^{2+}]_i$ increases triggered by muscarinic receptor activation. Together, the data

Table 2

CELL SPECIFICITY OF MUSCARINIC RECEPTORS

Cell Line	Cell Type	$[Ca^{2+}]_i$ (nM)	
		Resting	[CBC]-evoked
*1321N1	human astrocytoma	177 ± 12	230 ± 18
C6BU-1	rodent glial	169 ± 18	167 ± 5
*MDCK	canine kidney epithelial	189 ± 36	469 ± 13
3T3	rat fibroblast	196 ± 15	203 ± 12
NG108-15	neuroblastoma x glioma (differentiated)	166 ± 12	176 ± 27

*$p < 0.05$

Various cell lines were evaluated for muscarinic receptor function using 100 μM carbachol. $[Ca^{2+}]_i$ values were calculated as described in Methods. Values represent means ± S.E. of 3 - 9 separate experiments.

suggest that Ca^{2+} release is mediated via muscarinic but not nicotinic receptors, from internally sequestered Ca^{2+} stores rather than through plasma membrane ion channels.

Cell Specificity of Muscarinic Receptors: Several cell lines of different origin were examined for their Ca^{2+} response to muscarinic receptor agonists (Table 2). Like 1321N1 cells, MDCK cells produced an atropine-sensitive response to carbachol. In contrast, 3T3 and C6BU-1, showed no change in $[Ca^{2+}]_i$ after carbachol similar to DDT_1, a smooth muscle cell, which however, responded to norepinephrine (not shown), suggesting that α_1-adrenergic receptors in DDT_1 are coupled to Ca^{2+} mobilization. Carbachol produced no response in differentiated NG108-15 cells; however, depolarization (50 mM KCl) elevates $[Ca^{2+}]_i$ 3-fold through DHP-sensitive VOC in these cells (14,20).

Discussion

Stimulation of muscarinic receptors on 1321N1 cells results in marked increases in PI breakdown and Ca^{2+} mobilization (5). This study examines the mechanism of the latter functional response, and pharmacologically characterizes muscarinic receptors using quin 2. Ca^{2+} mobilization was induced by several full muscarinic agonists (acetylcholine > methacholine > carbachol > muscarine) and inhibited by muscarinic receptor antagonists (Table 1). The low potency of pirenzepine for the antagonism of Ca^{2+} release is similar to its potency for antagonism of the PI response, and thus confirms the suggestion that PI-coupled muscarinic receptors in 1321N1 cells cannot be characterized as "M_1" (22). Pirenzepine also blocks PI turnover with low affinity in CNS and peripheral tissues (21,22) as well as other cells (23).

In contrast to muscarinic receptor antagonists, even high concentrations of Ca^{2+} channel antagonists, metal cations, TTX and TEA all failed to block carbachol-induced Ca^{2+} mobilization. Conversely, Ca^{2+} channel agonists (BAY K8644), which enhance depolarization-sensitive Ca^{2+} influx (14), did not stimulate carbachol-induced Ca^{2+} release (not shown). The Ca^{2+} response was also observed in Ca^{2+}-free EGTA containing buffers, suggesting that $[Ca^{2+}]_{ex}$ was not required for Ca^{2+} mobilization, and again, TTX, TEA and La^{3+} had no effect on this response (not shown). Depolarization with KCl did not induce Ca^{2+} entry into 1321N1 cells (Fig. 1). This failure to detect a signal is not due to an inhibitory effect of quin 2 on the function of VOC, since differentiated NG108-15 cells, in parallel, produced a robust high $[K^+]_o$-dependent DHP-sensitive Ca^{2+} signal, which has also been observed in other quin-2 loaded cultured cell systems (24,25).

From these findings we suggest that muscarinic receptor agonists trigger Ca^{2+} release from intracellular Ca^{2+} stores rather than by stimulating external Ca^{2+} ion entry. Similar findings have been reported for the action of mitogenic lectins on lymphocytes (26) and growth factors on human fibroblasts (27). In contrast, two distinct mechanisms have been invoked for Ca^{2+} release following α_1-adrenergic receptor activation in smooth muscle cells (28,29). $[Ca^{2+}]_i$ levels were increased both due to extracellular Ca^{2+} influx as well as to intracellular Ca^{2+} mobilization. In 1321N1 cells, it appears that muscarinic receptor-coupled PI hydrolysis leads to Ca^{2+} mobilization predominantly from intracellular storage sites.

Acknowledgements

We appreciate Andrea Patton's assistance with cell cultures and the secretarial skills of Donna Partoza and Cheryl Sowards.

References

1. Meeker, R.B., and Harden, T.K. (1982) Mol. Pharmacol. 22: 310-319.
2. Hughes, A.R., Martin, M.W., and Harden, T.K. (1984) Proc. Natl. Acad. Sci. U.S.A. 81: 5680-5684.
3. Meeker, R.B., and Harden, T.K.. (1983) Mol. Pharmacol. 23: 384-392.
4. Gross, R.A., and Clark, R.B. (1977) Mol. Pharmacol. 13: 242-250.
5. Masters, S.B., Harden, T.K., and Brown, J.H. (1984) Mol. Pharmacol. 26: 149-155.
6. Evans, T., Smith, M.M., Tanner, L.I., and Harden, T.K. (1984) Mol. Pharmacol. 26: 395-404.
7. Orellana, S.A., Solski, P.A., and Brown, J.H. (1985) J. Biol. Chem. 260: 5236-5239.
8. Noronha-Blob, L., Richard, C., Kinnier, W.J., and U'Prichard, D.C. (1986) The Pharmacologist. 28 (3): 237.
9. Klagsbrun, M. (1980) J. Cell Biol. 84: 808-814.
10. Noronha-Blob, L., Lowe, V.C., Kinnier, W.J., and U'Prichard, D.C. (1986) Mol. Pharmacol. 30: 526-536.
11. Tsien, R.Y., Pozzan, T., and Rink, T.J. (1982) J. Cell Biol. 94: 325-334.
12. Hesketh, T.R., Smith, G.A., Moore, J.P., Taylor, M.V., and Metcalfe, J.C. (1983) J. Biol. Chem. 258: 4876-4882.
13. SAS Institute Inc. (1985) SAS User's Guide: Statistics, Version 5 Edition. Cary N.C.

14. Richard, C. L., U'Prichard, D.C., and Noronha-Blob, L. (1986) Fed. Proc. 45: (3), 513.
15. Rink, T.J., Smith, S.W., and Tsien, R.Y. (1982) Febs. Lett. 148: 21-26.
16. Charest, R., Blackmore, P.F., Berthron, B., and Exton, J.H. (1983) J. Biol. Chem. 258: 8769-8773.
17. White, J.R., Naccachi, P.M., Molski, T.F.P., Borgeat, T., and Shaafi, R.I. (1983) Biochem. Biophys. Res. Comm. 113: 44-50.
18. Evans, T., Hepler, J.R., Masters, S.B., Brown, J.H., and Harden, T.K. (1985) Biochem. J. 232: 751-757.
19. Noronha-Blob, L., Ferkany, J., Costello, D., Lowe, V., Rzeszotarski, J., Kinnier, W.J., and U'Prichard, D.C. (1986) Trends in Pharmacological Sciences, Supplement: p84.
20. Freedman, S.S., Dawson, G., Villereal, N.L., and Miller, R.J. (1984) J. Neurosci. 4: 1453-1467.
21. Lowe, V.C., Hanson, R.C., U'Prichard, D.C., and Noronha-Blob, L. (1987) Life Sci. in press.
22. Fisher, S.K., and Bartus, R.T. (1985) J. Neurochem. 45: 1085-1095.
23. Brown, J.H., Goldstein, D., and Masters, S.B. (1985) Mol. Pharmacol. 27: 525-531.
24. Albert, P.R., and Tashjian, A.H. (1984) J. Biol. Chem. 259: 15350-15363.
25. DiVirgilio, F., Pozzan, T., Wollheim, C.B., Vincentini, L.M., and Meldolesi, J. (1986) J. Biol. Chem. 261: 32-35.
26. Tsien, R.Y., Pozzan, T., and Rink, T.J. (1982) Nature (Lond) 295: 68-71.
27. Moolenaar, W.H., Tertoolen, L.G.J., and deLaat, S.W. (1984) J. Biol. Chem. 259: 8066-8069.
28. Reynolds, E.E., and Dubyak, G.R. (1985) Biochem. and Biophys. Res. Commun. 130 (2): 627-632.
29. Reynolds, E.E., and Dubyak, G.R. (1986) Biochem. and Biophys. Res. Commun. 136 (3): 927-934.