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RECEPTOR-MEDIATED REGULATION OF CALCIUM MOBILIZATION AND CYCLIC GMP SYNTHESIS IN NEUROBLASTOMA CELLS

Shunji Ohsako and Takeo Deguchi

Department of Medical Chemistry,
Tokyo Metropolitan Institute for Neurosciences,
2-6, Musashidai, Fuchu-city, Tokyo 183 Japan

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SUMMARY: In neuroblastoma N1E 115 cells, carbachol, histamine and PGE_1 elevated cyclic GMP content and, induced the efflux of preloaded $^{45}Ca^{2+}$, the release of membrane-bound Ca^{2+} measured by fluorescent CTC, and the increase in $[Ca^{2+}]_i$ as measured by Quin 2 fluorescence. The time course of the responses, the absolute requirement of extracellular Ca^{2+}, the inhibition by receptor blockers, and the concentration dependency on histamine were all similar between these responses. The observation indicates that the mobilization of Ca^{2+}, especially the increase of $[Ca^{2+}]_i$, may be intimately linked to the synthesis of cyclic GMP in the cells.

It has been shown that extracellular Ca^{2+} was required for the receptor-mediated elevation of cyclic GMP in a variety of cells (1). The omission of extracellular Ca^{2+} partially or completely abolished the elevation of cyclic GMP by neurotranmitters or hormones depending on preincubation conditions without Ca^{2+} (2-6). Ca^{2+} ionophore A23187 which promoted the passive transport of Ca^{2+} across biological membranes also elevated intracellular cyclic GMP contents (6-8). Although the essential role of Ca^{2+} for the elevation of cyclic GMP has been established, the exact correlation between Ca^{2+} mobilization and cyclic GMP synthesis in response to neurotransmitter stimulation has remained to be established. The uptake of extracellular $^{45}Ca^{2+}$ was not observed by the agents that increased intracellular cyclic GMP (8). On the other hand, we and other laboratories

Abbreviations: PGE_1, prostaglandin E_1; CTC, chlortetracycline; Quin 2, 2-[[2[bis(carboxymethyl)amino[-5-methylphenoxy]methyl]-6-methoxy-8-[bis(carboxymethyl)amino]quinoline; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; DMEM, Dulbecco-Vogt modified Eagle's minimal essential medium; HEPES, 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis (b-aminoethyl ether) N,N,N',N'-tetraacetic acid; CCCP, carbonyl cyanide m-chlorophenylhydrzone.

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have shown that cholinergic agents stimulated the efflux of $^{45}Ca^{2+}$ from preloaded adrenal chromaffin and pancreatic acinar cells (8-10). To further investigate the correlation between Ca^{2+} mobilization and cyclic GMP synthesis, we used neuroblastoma N1E 115 cells for its marked responsiveness of cyclic GMP and for its homogeneity (4, 5, 11, 12), In this paper we report that the efflux of $^{45}Ca^{2+}$, the release of membrane-bound Ca^{2+} as measured by CTC fluorescense, and $[Ca^{2+}]_i$ measured by Quin 2 changed in parallel with the change of cyclic GMP contents.

EXPERIMENTAL PROCEDURES

Culture of neuroblastoma cells--Neuroblastoma N1E 115 cells were grown in Nunc plastic dishes or Falcon plastic flasks in DMEM supplemented with 5% fetal bovine serum (Gibco, Grand Island, NY) in humidified atmosphere of 10% CO_2 and 90% air at 37°C (12). Cells were usually grown for 7 days to confluence prior to use.

Measurement of $^{45}Ca^{2+}$ efflux--Cells cultured in dishes were preincubated in 1 ml of DMEM supplemented with 5% fetal bovine serum and 2 μCi of $^{45}CaCl_2$ overnight (9). After the cells were rapidly washed three times with 2 ml of HEPES-buffered DMEM, one ml of HEPES-buffered DMEM containing carbachol, histamine or PGE_1 was added to the cells. After the incubation time indicated, the medium was transferred into a test tube in ice and centrifuged at 1500 X g for 15 min. The radioactivity in the supernatant was determined by a liquid scintillation counter.

Measurement of CTC fluorescence--Cells were detached from flasks and washed twice with HEPES-buffered saline without Ca^{2+} and Mg^{2+} followed by centrifugation at 200 X g for 5 min. HEPES-bufferd saline consisted of 145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 5 mM glucose, and 10 mM HEPES (pH 7.4). The cells were suspended in HEPES-buffered saline without Ca^{2+} and Mg^{2+} (5-10 X 10^5 cells/ml) and transferred into a quartz cuvette in Hitachi 650-10 S spectrofluorometer equipped with a magnetic stirrer and a thermostatted cell holder. Two min after the addition of CTC (20 μM, Sigma), Ca^{2+} (1 mM) or EGTA (1 mM) was added to cell suspensions equilibrated at 37°C. The cells were incubated for 30 min which was sufficient for the fluorescence to reach a steady state level. Then the agent to be tested was added and the fluorescence was monitered on a recorder. The excitation and emission wavelengths were set at 390 nm (10 nm slit) and 520 nm (10 nm slit), respectively (13, 14).

Measurement of $[Ca^{2+}]_i$ by a fluorescent Ca^{2+} indicater, Quin 2--Cells (5-10 X 10^5 cells/ml) were suspended in DMEM supplemented with 5% fetal bovine serum and incubated with 100 μM Quin 2 acetoxymethyl ester (Dojindo Laboratories) for 15 min at 37°C. Cells were diluted 5-fold and further incubated for 1 h. After loading of Quin 2, the cells were washed twice with HEPES-buffered saline or Ca^{2+}-free HEPES-buffered saline followed by centrifugation at 200 X g for 5 min and resuspended in respective salines. The fluorescence of Quin 2 loaded neuroblastoma cells was measured at an excitation wavelength of 339 nm (10 nm slit) and an emission wavelength of 500 nm (10 nm slit) using spectrofluorometer as described under "Measurement of CTC fluorescence". $[Ca^{2+}]_i$ was calculated from fluorescence signals as described by Tsien et al. (15, 16).

Assay of cyclic GMP--Assay of cyclic GMP were performed as described (11). Protein was determined by the method of Lowry et al. (17).

RESULTS

To examine whether or not carbachol, histamine and PGE_1 which elevate the intracellular cyclic GMP contents in the neuroblastoma cells stimulate the influx of Ca^{2+}, the uptake of $^{45}Ca^{2+}$ by the cells was measured. There was no significant uptake of $^{45}Ca^{2+}$ into the cells by the agents (data not shown). On the other hand, these agents resulted in a rapid release of $^{45}Ca^{2+}$ from prelabelled cells within 15 s (Fig. 1). This rapid change in $^{45}Ca^{2+}$ efflux was comparable to the rapid increase in cyclic GMP contents (4, 5). $^{45}Ca^{2+}$ efflux induced by carbachol and histamine was blocked by atropine (10 μM) and pyrilamine (10 μM) (data not shown) indicating that the efflux of preloaded $^{45}Ca^{2+}$ was mediated by muscarinic and histaminic receptors, respectively. To examine whether the $^{45}Ca^{2+}$ efflux was a consequence of cyclic GMP elevation or vice versa, cyclic GMP (1 mM), dibutyryl cyclic GMP (1 mM) or nitroprusside which elevated intracellular cyclic GMP levels (18, 19) was added to neuroblastoma cells. These agents failed to stimulate the efflux of preloaded $^{45}Ca^{2+}$ (data not shown) indicating that cyclic GMP elevation may be not a causal event to $^{45}Ca^{2+}$ efflux.

Two pools are conceivable as to the sources of $^{45}Ca^{2+}$ released from preloaded cells: membrane-bound Ca^{2+} and cytosolic Ca^{2+}. To study this

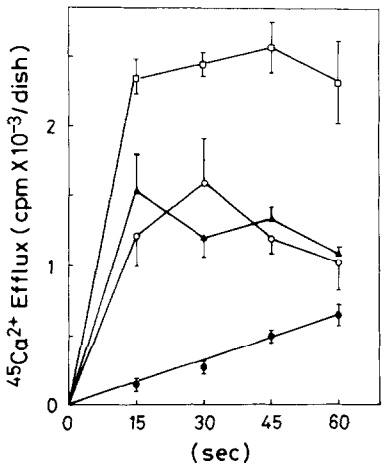


Fig. 1. Effect of carbachol, histamine and PGE_1 on the efflux of Ca^{2+} from preloaded neuroblastoma N1E 115 cells. Cells were incubated for the indicated time in the absence (●) or in the presence of 1 mM carbachol (○), 0.1 mM of histamine (□), or 10 μM of PGE_1 (▲). Points and bars indicate the means and S.E. of 4 or 5 samples.

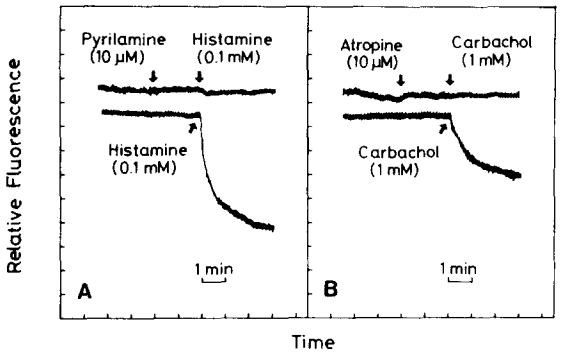


Fig. 2. Effect of histamine and carbachol on the steady-state fluorescence of cell-associated CTC. Incubation of neuroblastoma cells and measurement of CTC fluorescence were performed as described under "EXPERIMENTAL PROCEDURES". Histamine (0.1 mM), pyrilamine (10 μM), carbachol (1 mM) and atropine (10 μM) were added at arrows.

point, CTC, a fluorescent probe that forms highly fluorescent complexes with Ca^{2+} and Mg^{2+} bound to membrane, was employed (13, 14). The addition of histamine (0.1 mM) and carbachol (1 mM) caused a rapid decrease in fluorescence of cell-associated CTC (Fig. 2). Pyrilamine (10 μM) and atropine (10 μM) blocked the decrease in fluorescence induced by histamine (0.1 mM) and carbachol (1 mM), respectively, indicating that the changes in cell-associated CTC fluorescence induced by these agents were mediated by their specific receptors. PGE_1 (10 μM) also caused a rapid decrease of CTC fluorescence (data not shown).

It could be that a portion of membrane-bound Ca^{2+} was liberated into medium, while another portion was taken up by the cells to result in an increase of $[Ca^{2+}]_i$ upon stimulation by these agents. Therefore, $[Ca^{2+}]_i$ was measured by use of fluorescent Ca^{2+} indicator Quin 2 (15, 16). Addition of histamine (0.1 mM), carbachol (1 mM) and PGE_1 (10 μM) to cell suspensions rapidly increased $[Ca^{2+}]_i$ from a basal level of 100 nM to 150, 110 and 125 nM, respectively, and declined to the basal level within 2 min (Fig. 3, data for PGE_1 was not shown). This time course was comparable to that of cyclic GMP elevation induced by the agents. The increase in $[Ca^{2+}]_i$ induced by histamine and carbachol was also blocked by pyrilamine and atropine, respectively, indicating that the increases in $[Ca^{2+}]_i$ were receptor-mediated

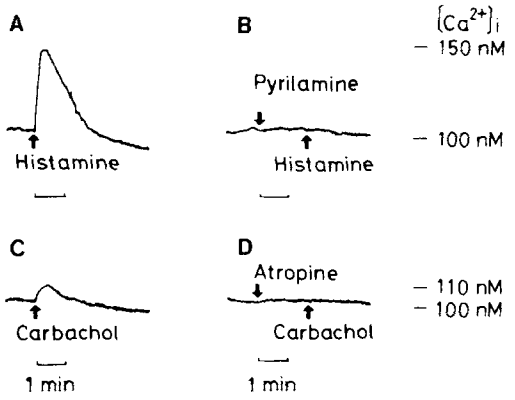


Fig. 3. Effect of histamine and carbachol on $[Ca^{2+}]_i$ in neuroblastoma cells as indicated by Quin 2 fluorescence. Incubation of neuroblastoma cells and measurement of $[Ca^{2+}]_i$ with Quin 2 were performed as described under "EXPERIMENTAL PROCEDURES". Histamine (0.1 mM), pyrilamine (10 μM), carbachol (1 mM) and atropine (10 μM) were added at arrows.

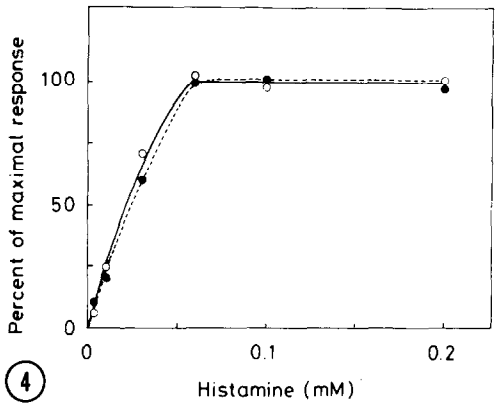
responses. The response of $[Ca^{2+}]_i$ by carbachol was smaller than that of histamine, which was compatible with a smaller increase in cyclic GMP contents by carbachol than histamine (data not shown).

To examine the correlation between the increase in $[Ca^{2+}]_i$ and cyclic GMP synthesis, the effect of varying concentrations of histamine on the change in Quin 2 fluorescence and cyclic GMP contents was determined. There was a maximal response of Quin 2 fluorescence and cyclic GMP synthesis with 60 μM of histamine and a half-maximal change with approximately 20 μM (Fig. 4).

Both the decrease in CTC fluorescence and the increase in $[Ca^{2+}]_i$ by histamine were dependent on extracellular Ca^{2+}. In Ca^{2+}-free saline containing EGTA, both changes were completely abolished (Fig. 5). In another experiment, cells were preincubated in Ca^{2+}-free saline containing EGTA for various periods. Both of the cyclic GMP synthesis and the increase in $[Ca^{2+}]_i$ were gradually diminished and completely abolished after 30 min of preincubation (data not shown).

DISCUSSION

The present study shows that carbachol, histamine and PGE_1 induces the efflux of $^{45}Ca^{2+}$, the release of membrane-bound Ca^{2+} measured by



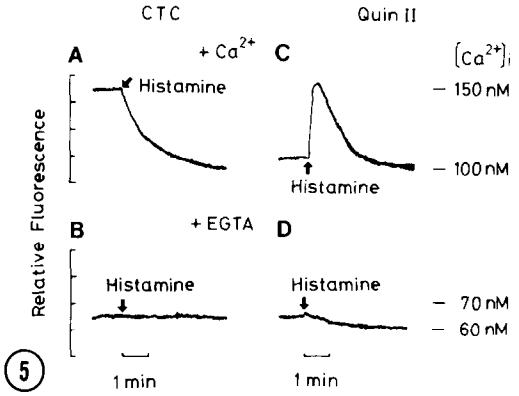


Fig. 4. Effect of histamine concentration on the response of Quin 2 fluorescence and cyclic GMP contents in neuroblastoma cells. Peak change in Quin 2 fluorescence (●) and cyclic GMP content at 30 s (○) after the addition of histamine were plotted as % of maximal response. Each point was the mean of 2 and 4 samples for the change in Quin 2 fluorescence and cyclic GMP content, respectively.

Fig. 5. Effect of removal of extracellular Ca^{2+} on the responses of CTC and Quin 2 fluorescences to histamine. Cells were preincubated in HEPES-buffered saline or Ca^{2+}-free HEPES-buffered saline containing 1 mM EGTA at 37°C for 30 min. Histamine (0.1 mM) was added at arrows.

fluorescent CTC, and $[Ca^{2+}]_i$ elevation measured by the fluorescent Ca^{2+} indicator Quin 2 in neuroblastoma N1E 115 cells. All of these responses induced by carbachol and histamine were blocked by their receptor blockers in the same fashion as the elevation of cyclic GMP. The time course, the absolute requirement of extracellular Ca^{2+}, and the concentration dependency on histamine were all similar with the cyclic GMP synthesis, the release of preloaded $^{45}Ca^{2+}$, the decrease in cell-associated CTC fluorescence, and the increase in $[Ca^{2+}]_i$, indicating that these processes are intimately correlated with each other. The uncoupling agent CCCP, which is expected to release mitochondrial Ca^{2+} store (20), did not increase cyclic GMP content in the cells (data not shown). Therefore, a cellular Ca^{2+} pool which was equilibrated with extracellular Ca^{2+} may be directly involved in the synthesis of cyclic GMP. The membrane-bound Ca^{2+} might be a such physiologically important pool. Ca^{2+} store in membrane could be partly liberated into medium, which was reflected in the release of $^{45}Ca^{2+}$, while another portion of membrane-bound Ca^{2+} was taken up into the cells to increase $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ thus elevated may induce the activation of guanylate cyclase, possibly by collaborating with L-arginine as previouly demonstrated by us (21). Although there were some discrepancies in the sensitivity of CTC fluorescence on extracellular Ca^{2+} between neuroblastoma cells, pancreatic acinar cells, and neutrophils, this might represent the difference in Ca^{2+} pools in various cells (13, 14). In conclusion, Ca^{2+} mobilization, especially the elevation of $[Ca^{2+}]_i$ in response to transmitter stimulation, might be directly linked to the synthesis of cyclic GMP in neuroblastoma N1E 115 cells.

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