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Galanin Increases Cytoplasmic Calcium in Insulin-Producing RINm5F Cells by Activating Phospholipase C

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We showed previously that the neuropeptide, galanin, transiently and promptly increases the cytoplasmic concentration of Ca^{2+}, $[Ca^{2+}]_i$, in insulin producing clonal RINm5F cells prior to a reduction in $[Ca^{2+}]_i$. In this study, we examined the mechanisms underlying the increased $[Ca^{2+}]_i$. We found that galanin (100 nM) transiently increased the $[Ca^{2+}]_i$ in the presence of 15 mM glyceraldehyde and 3.3 mM glucose. This effect was abolished by both the inhibitor of the microsomal Ca^{2+} ATPase, thapsigargin, which depletes the intracellular Ca^{2+} stores, and the specific inhibitor of phospholipase C, U73122. In contrast, the blocker of L-type Ca^{2+} release, ryanodine, did not affect galanin-induced increase in $[Ca^{2+}]_i$. Thus, galanin induces a rapid mobilization of Ca^{2+} from intracellular Ca^{2+} stores in insulin producing RINm5F cells by an effect mediated by activated phospholipase C.

The neuropeptide, galanin, inhibits insulin secretion from pancreatic islets (1,2) as well as from insulin producing clonal RINm5F cells (3). The mechanism underlying this action of the neuropeptide has been thought to involve inhibition of adenylate cyclase (4), activation of ATP-regulated K^+ channels with subsequent hyperpolarization-induced closure of voltage-dependent Ca^{2+} channels and lowering of the cytoplasmic concentration of Ca^{2+}, $[Ca^{2+}]_i$, (1,3,5) as well as an inhibitory effect on the exocytosis process (6). The effects of galanin are, furthermore, mediated by G proteins (7).

Beside these actions of galanin, we previously showed that the neuropeptide also transiently raises the $[Ca^{2+}]_i$ in RINm5F cells (8). This surprising effect was later confirmed by Lang et al. who also suggested that it is mediated by a G_i-protein, since it was abolished by pertussis toxin (9). This suggestion is corroborated by another recent finding that binding of galanin to RINm5F cell membranes is reduced by antibodies against $G\alpha_{i1}/\alpha_{i2}$ (10). Hypothetically, therefore, galanin could activate these G proteins, causing formation of phospholipase C and induction of inositol 1,4,5-trisphosphate (IP_3)-mediated mobilization of Ca^{2+} from intracellular Ca^{2+} stores. Other actions of galanin, such as the closure of membraneous Ca^{2+} channels leading to reduction in $[Ca^{2+}]_i$ and inhibition of insulin secretion, could then be the result of interaction with other G-proteins (cf. 7).

Since the mechanism underlying the galanin-induced transient increase in $[Ca^{2+}]_i$ in RINm5F cells is not established, we have in the present study further examined the effect. Since we show that the effect is abolished by the phospholipase C inhibitor, U73122, and the inhibitor of microsomal Ca^{2+} ATPase, thapsigargin, which depletes IP_3 sensitive intracellular Ca^{2+} stores (11), we substantiate the hypothesis that the neuropeptide in this insulin producing clonal cell line increases $[Ca^{2+}]_i$ by activating phospholipase C.

MATERIALS AND METHODS

Materials. Cells of the rat insulinoma cell line, RINm5F, were cultured in a RPMI-1640 medium (Gibco BRL, Paisley, Scotland) supplemented with 10% fetal calf serum (Biological Ind, Kibbutz Beit Haemek, Israel), 100 IU/ml penicillin, 100 μg/ml streptomycin (both Biological Ind) and 2.5 μg/ml amphotericin B (Gibco) at 37°C in an athmosphere of humified air. Cells were subcultured every 4–5 days by trypsination and the medium was changed the day after each subculture and thereafter every second day. Cells were used for experiments after reaching confluency at 4–7 days after subculture.

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Preparation of cells for experiments. Cells were trypsinated and thereafter allowed to recover at a concentration of 10^6 cells/ml for 2 hours in 10 ml of RPMI-1640 medium supplemented with 10% fetal calf serum at 37°C. During this period, the cells were continuously shaken to avoid attachment. The cells were thereafter loaded with FURA-2AM (1 μM; Sigma Chemical Co, St Louis, MO, USA) for another 45 min, washed three times in a Hepes medium (25 mM Hepes; 125 mM NaCl; 5.9 mM KCl, 1.28 mM $CaCl_2$; 1.1 mM $MgCl_2$) and allowed to equilibrate for 20 min in the Hepes medium at room temperature. The cells were washed again, counted in a Bürker chamber, resuspended in the Hepes medium at a concentration of 0.5×10^6 cells/ml and transferred to a cuvette for measurement of $[Ca^{2+}]_i$.

Measurement of $[Ca^{2+}]_i$. The cytoplasmic calcium concentration $[Ca^{2+}]_i$ was measured by dual wavelenght spectrophotofluorometry using FURA-2AM as indicator and a Perkin-Elmer LS-50 spectrophotofluorometer. The excitation wavelength was continuously altered between of 340 and 380 nm and the emission wavelength was 510 nm. The cell suspension was continuously stirred with a stirrbar mounted at the side of the cuvette. D-glyceraldehyde (Sigma), synthetic rat galanin (Peninsula Labs, Merseyside, U.K.), carbachol, nifedipine, thapsigargin, ryanodine (all Sigma) or 1-[6-[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U-73122) (Research Biochemicals Int., Natick, MA, U.S.A.) was added to the cuvette in μl volumes from stock solutions of high concentrations at specific time points as indicated in the Figures. At 1,200 to 1,600 seconds after start of experiment, KCl (20 mM) was always added to verify an increased $[Ca^{2+}]_i$. Finally, at the end of each experiment, fluorescence maximum was obtained by adding 0.05% Triton X-100 and fluorescence minimum was obtained by adding [ethylene-bis(oxyethylenenitrilo)]tetraacetic acid (EGTA; Sigma) in excess to the cell suspensions. The $[Ca^{2+}]_i$ was calculated according to the previously described formula (12). The K_d was assumed to be 224 nM.

Statistics. Results are shown as representative traces of 3–9 observations for each individual experimental series. The galanin-induced increase in $[Ca^{2+}]_i$ was calculated from the baseline level of $[Ca^{2+}]_i$ before addition of galanin. Control traces were always run the same day from the same cells. Statistical analysis was performed by the Students t-test for paired or unpaired observations.

RESULTS

Effects of galanin on $[Ca^{2+}]_i$ *in RINm5F cells.* Fig. 1 shows that D-glyceraldehyde (15 mM) in the presence of 3.3 mM glucose gradually increased the $[Ca^{2+}]_i$ when added to the RINm5F cell suspension, which is a well known effect of glyceraldehyde (8). After reaching a plateau in $[Ca^{2+}]_i$, galanin (100 nM) was added, which promptly albeit transiently increased the $[Ca^{2+}]_i$. The peak of the galanin-induced $[Ca^{2+}]_i$ peak was 118±26 nM ($n = 9$, $p<0.001$). After the peak, $[Ca^{2+}]_i$ transiently decreased to values below the prestimulus values.

Effects of nifedipine on galanin-induced increase in $[Ca^{2+}]_i$. To study whether the galanin-induced increase in $[Ca^{2+}]_i$ is evident also without any uptake of extracellular Ca^{2+}, we introduced

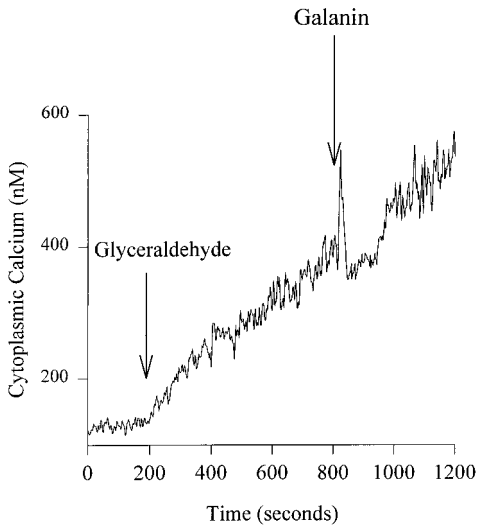

FIG. 1. Cytoplasmic Ca^{2+} concentration in cell suspensions of RINm5F cells in a Hepes medium at 3.3 mM glucose. D-glyceraldehyde (15 mM) and rat galanin (100 nM) were added as indicated. A representative trace of 9 separate experiments is shown.

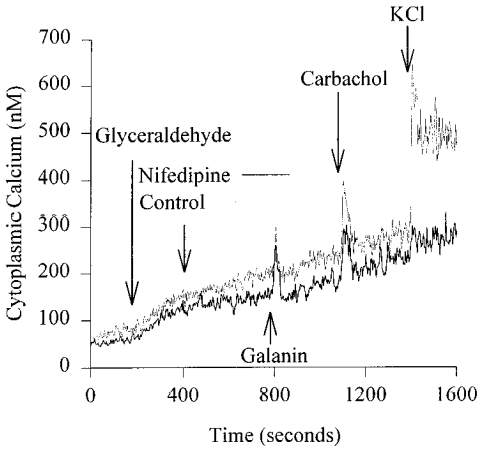

FIG. 2. Cytoplasmic Ca^{2+} concentration in cell suspensions of RINm5F cells in a Hepes medium at 3.3 mM glucose. D-glyceraldehyde (15 mM), rat galanin (100 nM), carbachol (0.1 mM) and KCl (20 mM) were added as indicated. In one series, nifedipine (1 μM) was added at 400 seconds. A representative trace of 3 separate experiments of each series is shown.

the inhibitor of membraneous L-type Ca^{2+} channels, nifedipine (1 μM), prior to galanin. Fig. 2 shows that nifedipine failed to affect the galanin-induced increase in $[Ca^{2+}]_i$. The galanin-induced peak in $[Ca^{2+}]_i$ was 137±39 nM without nifedipine versus 119±26 nM in the presence of nifedipine in experiments run in parallel ($n = 3$; n.s.). Similarly, the cholinergic agonist, carbachol (0.1 mM), increased the $[Ca^{2+}]_i$ also in the presence of nifedipine, whereas the increase in $[Ca^{2+}]_i$ induced by depolarization by KCl was abolished.

Effects of thapsigargin on galanin-induced increase in $[Ca^{2+}]_i$. Thapsigargin inhibits the microsomal Ca^{2+} ATPase which depletes the intracellular Ca^{2+} stores (11). Fig. 3 shows that thapsigargin increased $[Ca^{2+}]_i$, and thereafter both galanin and carbachol failed to increase the $[Ca^{2+}]_i$. Hence, depleting the intracellular Ca^{2+} stores abolished the galanin-induced increase in $[Ca^{2+}]_i$.

Effects of U73122 and ryanodine on galanin-induced increase in $[Ca^{2+}]_i$. U73122 inhibits phospholipase C (13,14) and ryanodine inhibits Ca^{2+}-induced Ca^{2+}-release from intracellular Ca^{2+}

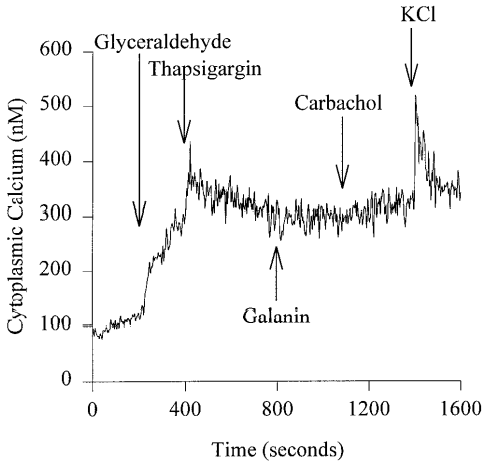

FIG. 3. Cytoplasmic Ca^{2+} concentration in cell suspensions of RINm5F cells in a Hepes medium at 3.3 mM glucose. D-glyceraldehyde (15 mM), thapsigargin (2 μM), rat galanin (100 nM), carbachol (0.1 mM) and KCl (20 mM) were added as indicated. A representative trace of 4 separate experiments is shown.

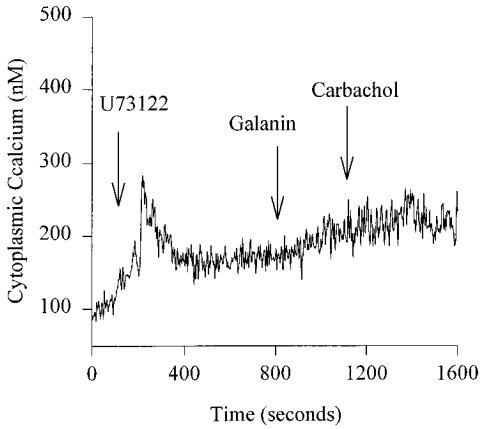

FIG. 4. Cytoplasmic Ca^{2+} concentration in cell suspensions of RINm5F cells in a Hepes medium at 3.3 mM glucose. The inhibitor of phospholipase C, U73122 (5 μM), rat galanin (100 nM) and carbachol (0.1 mM) were added as indicated. A representative trace of 3 separate experiments is shown.

stores (15). Fig. 4 shows that U73122 completely abolished both galanin- and carbachol-induced increase in $[Ca^{2+}]_i$ whereas Fig. 5 shows that ryanodine was without effect since the galanin-induced peak in $[Ca^{2+}]_i$ in the presence of ryanodine was 101±16 nM versus 111±12 nM in the absence of ryanodine in experiments run in parallel (n = 3; n.s.). Hence, the results suggest that galanin increases $[Ca^{2+}]_i$ in RINm5F cells by stimulating phospholipase C.

DISCUSSION

In this study, we confirm that galanin promptly and transiently increases $[Ca^{2+}]_i$ when added to insulin producing RINm5F cells before the levels are reduced to values below prestimulus levels (8,9). This action of galanin is not seen in normal B cells (1,5,16). Hence, different signaling pathways coupled to the galanin receptors seem to have evolved in different insulin producing cells. In contrast, however, galanin has been shown to raise the $[Ca^{2+}]_i$ in clonal growth hormone producing cells, GH_3/B6 cells (17), showing that galanin receptors couple to events increasing $[Ca^{2+}]_i$ in some cells, but not in others. It should also be emphasized, that galanin does not increase

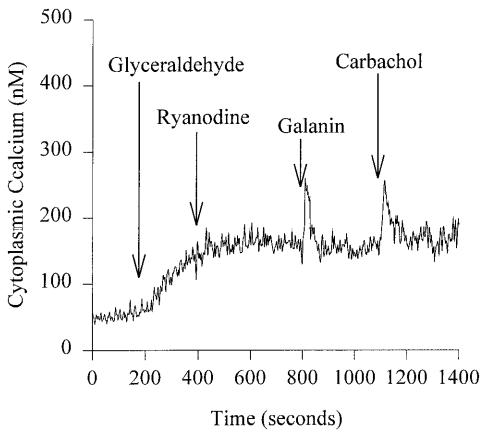

FIG. 5. Cytoplasmic Ca^{2+} concentration in cell suspensions of RINm5F cells in a Hepes medium at 3.3 mM glucose. D-glyceraldehyde (15 mM), ryanodine (0.1 mM), rat galanin (100 nM) and carbachol (0.1 mM) were added as indicated. A representative trace of 3 separate experiments is shown.

the $[Ca^{2+}]_i$ in all sublines of RINm5F cells (18), showing that expression of different signaling mechanisms have evolved in different subclones of RINm5F cells.

An increased $[Ca^{2+}]_i$ might be due to stimulated uptake of extracellular Ca^{2+} and/or to stimulated mobilization of Ca^{2+} from intracellular Ca^{2+} stores (19). Our finding that nifedipine, which inhibits L-type Ca^{2+} from intracellular Ca^{2+} stores (19). Our finding that nifedipine, which inhibits L-type Ca^{2+} channels (20), did not affect the galanin-induced increase n $[Ca^{2+}]_i$, indicates that galanin, as carbachol, does not increase $[Ca^{2+}]_i$ by inducing uptake of extracellular Ca^{2+}. Furthermore, our finding that thapsigargin, which depletes Ca^{2+} from the stores (11), prevented both galanin and carbachol from increasing $[Ca^{2+}]_i$ suggests that the increase in $[Ca^{2+}]_i$ induced by both galanin and carbachol is due to release of the cation from its intracellular stores.

Mobilization of Ca^{2+} from intracellular Ca^{2+} stores might be mediated by IP_3 formed through activation of phospholipase C (19,21) or by Ca^{2+}-induced Ca^{2+}-release mediated by activation of the ryanodine receptors (15,22), which is a process that might be activated by cyclic ADP-ribose (23). We found that the increase in $[Ca^{2+}]_i$ induced by both galanin and carbachol was totally abolished by the inhibitor of phospholipase C, U73122, but unaffected by the inhibitor of Ca^{2+}-induced Ca^{2+}-release, ryanodine. This suggests a role for IP_3 generated through activation of phospholipase C as the mediator of the galanin- and carbachol-induced increase in $[Ca^{2+}]_i$ in RINm5F cells.

Previously, galanin has been shown to inhibit phosphoinositide turnover in ventral hippocampal slices through an action involving impaired uptake of Ca^{2+} causing lowered $[Ca^{2+}]_i$ (24). In contrast, in the cardiac tissue of the mudpuppy, galanin has been shown to stimulate phosphoinositide turnover (25). In this context, it is of interest that galanin recently was reported to stimulate phosphoinositide turnover also in normal islets (26). The hypothesis that different cell types express different signaling mechanisms induced by galanin is corroborated by findings that galanin receptors of different cell types show different sensitivity to galanin antagonists suggesting the existence of at least two putative galanin receptors (GL-1 receptors in hippocampus and GL-2 receptors in insulin producing cells; 27).

In summary, besides reducing $[Ca^{2+}]_i$ by inducing hyperpolarization, galanin also transiently increases $[Ca^{2+}]_i$ through activation of phospholipase C in RINm5F cells. This dual response might be explained by two different G-proteins activated by galanin, the net effect being inhibited insulin secretion.

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