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INTERRELATIONSHIP BETWEEN INSULIN-LIKE GROWTH FACTOR I-INDUCED ACTIVATION OF THE Na^+/H^+-ANTIPORTER AND INTRACELLULAR Ca^{2+}-MOBILIZATION IN THYROID CELLS

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Insulin-like growth factor I (IGF-I) increased cytoplamic pH (pHi) and cytoplasmic Ca^{2+} ($[Ca^{2+}]i$) in cultured porcine thyroid cells. Inhibition of the Na^+/H^+-antiporter by dimethylamiloride or a reduction of external Na^+-concentrations attenuates the increases in pHi and $[Ca^{2+}]i$. The $[Ca^{2+}]i$ response to IGF-I is a pHi-dependent process. IGF-I activates Na^+/H^+-antiporter and alkalinizes thyroid cells. The resulting increase in pHi facilitates the $[Ca^{2+}]i$ response by adjusting the pHi closer to the pHi-optimum of the intracellular Ca^{2+}-mobilizing system. One of the biological functions of IGF-I-induced activation of the Na^+/H^+-antiporter is to shift the pHi to an optimal value for the $[Ca^{2+}]i$ response.

Insulin-like growth factor I (IGF-I) has been shown to activate the Na^+/H^+-antiporter in thyroid cells (1,2). However, in the mitogenic responses, the biological significances of the resulting increase in cytoplasmic pH (pHi) are still unclear. IGF-I also increases the cytoplasmic Ca^{2+} ($[Ca^{2+}]i$) and stimulates the thyroid cell proliferation (3). However, the interrelationship between the Na^+/H^+-antiporter and the intracelluar Ca^{2+}-mobilization has not been known. We intended to reveal this interrelationship. The data presented here indicate that intracellular Ca^{2+}-mobilization by IGF-I is a pHi-dependent process with a pHi-optimum around 7.2-7.4. Inhibition of the Na^+/H^+-antiporter attenuates the IGF-I-induced $[Ca^{2+}]i$ response. The activation of the Na^+/H^+-antiporter facilitates the Ca^{2+}-mobilization by shifting the pHi towards the optimum for the Ca^{2+}-mobilization.

MATERIALS AND METHODS

Thyroid cell culture: Thyroid cells were obtained from porcine thyroid glands as described previously (4). Freshly isolated cells were suspended ($3x10^6$

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cells/ml) in Eagle's minimum essential medium (MEM) supplemented with 0.5% human IGF-I-deficient serum and antibiotics (penicillin, 200 units/ml; streptomycin, 50 μg/ml). Cells were cultured as a suspension at 37°C in a 95% air: 5% CO_2 water-saturated atmosphere (5).

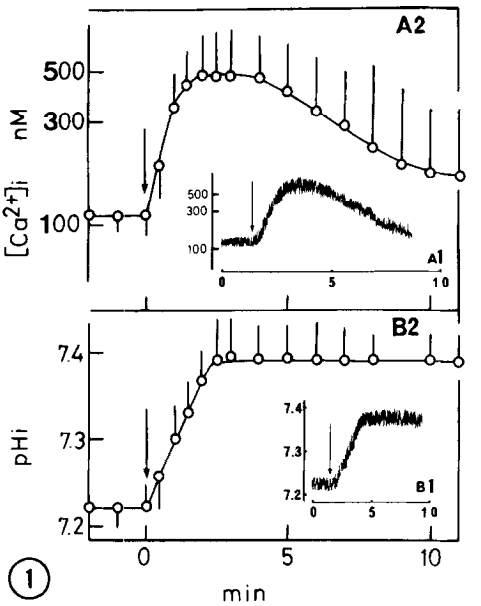
Cytoplasmic Ca^{2+} ($[Ca^{2+}]i$) measurement with fura-2: $[Ca^{2+}]i$ was measured with fura-2 (3,6). Porcine thyroid cells, cultured for 16 h, were washed twice in phosphate-buffered saline glucose (PBSG)-HEPES solution, consisting of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.45 mM $CaCl_2$, 0.49 mM $MgCl_2$, 5.6 mM glucose, and 10 mM HEPES (pH 7.4). The cells were resuspended in the same buffer containing 2 μM fura-2 AM and incubated for 30 min at 37°C. Under these conditions, fura-2 AM permeates the cells, is hydrolyzed to fura-2, and binds cytoplasmic free calcium. After loading, the cells were washed twice to remove extracellular dye and were resuspended at $3x10^6$ cells/ml in PBSG-HEPES (pH 7.4). The fura-2-loaded cells were put into a thermostatic cuvette (37°C) in a Hitachi F-4000 fluorometer (Hitachi, Tokyo). The cell suspension was continuously stirred with a magnetic microbar. After reaching a steady state, IGF-I was added to the cuvette using a syringe. Fluorescence was recorded with excitation and emission wavelengths of 340 and 505 nm respectively. Fluorescence was corrected for cell autoflurescence at 380 nm. The cells were subsequently lysed by adding Triton X-100 (0.3 %; final concentration) to obtain the signal of the calcium-saturated dye (Fmax). The signal (Fmin) from the Ca^{2+} free form of the dye was recorded by adding EGTA at pH 8.2 to a final concentration of 10 mM. The $[Ca^{2+}]i$ corresponding to an intracellular fura-2 fluorescence (F) was calculated by the equation: $[Ca^{2+}]i = Kd (F-Fmin/Fmax-F)$, using Kd = 224 nM according to Grynkiewicz et al. (6).

Cytoplasmic pH (pHi) measurement with BCECF: Cytoplasmic pH (pHi) was measured with 2',7'-bis(2-carboxyethyl)-5 (and 6-) carboxyfluorescein (BCECF) (7,8). Porcine thyroid cells, cultured for 16 h, were washed twice in PBSG-HEPES (pH 7.2). The cells were resuspended in the same buffer containing 2 μM BCECF-AM (tetraacetoxymethyl ester of BCECF), and incubated for 30 min at 37°C. BCECF-AM was converted to the impermeant BCECF by cytoplasmic esterases (15). After loading, the cells were washed twice to remove extracellular dye and were resuspended at $3x10^6$ cells/ml in PBSG-HEPES (pH 7.2). The BCECF-loaded cells were put into a thermostatic cuvette (37°C) in a Hitachi F-4000 fluorometer (Hitachi, Tokyo), and the cell suspension was continuously stirred with a magnetic microbar. After reaching a steady state, IGF-I was added to the cuvette using a microsyringe. The pHi-dependent emission intensity was continuously recorded (excitation wavelength 500 nm; emission wavelength 530 nm; slits 5.5/11-nm bandpass). Calibration of cytoplasmic dye fluorescence as a function of pHi was obtained from H^+ equilibration methods using the K^+/H^+ ionophore nigericin, which sets $[H^+]i/[H^+]o$ equal to $[K^+]i/[K^+]o$ as previously described (9). Briefly, calibration was obtained by incubation of BCECF-loaded cells in K^+ medium (140 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose) buffered with 10 mM HEPES, and containing nigericin (0.7 μg/ml). The pHi-dependent emission intensity was then measured.

Materials etc.: Recombinant human IGF-I was obtained from Amgen Biologicals (Thousand Oaks, CA, U.S.A.); dimethylamiloride, nigericin, and HEPES from Sigma (St. Louis, MO, U.S.A.); Eagle's MEM from Flow Laboratories (Irvine, Scotland, U.K.); fura-2 AM and BCECF-AM from Molecular Probes, Inc. (Junction City, OR, U.S.A.). Fura-2 AM and BCECF-AM were dissolved in dimethyl sulfoxide and stored at -60°C. IGF-I-deficient serum was obtained from a patient with pituitary dwarfism; the serum IGF-I concentration was less than 10 ng/ml. All other chemicals were of the highest purity available commercially. Experiments were conducted at least 6 times. Data were statistically analyzed with analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

RESULTS

The cells were loaded with fura-2. Addition of 100 ng/ml IGF-I resulted in a prompt increase in $[Ca^{2+}]i$ (Fig. 1A). The IGF-I-induced $[Ca^{2+}]i$ response



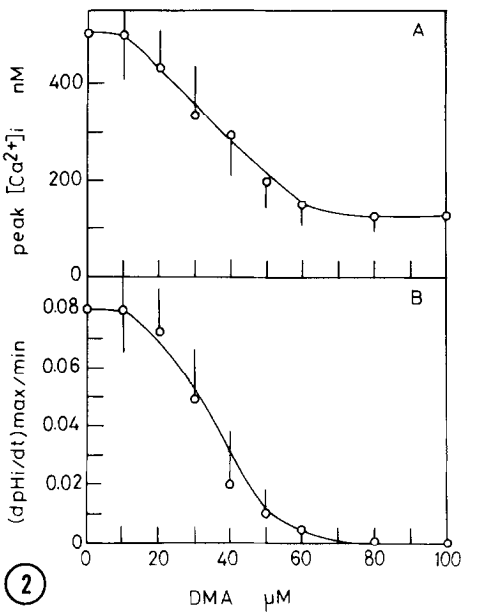
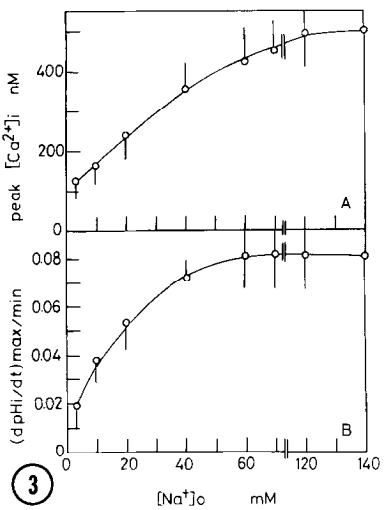


Fig. 1. Effect of insulin-like growth factor-I (IGF-I) on cytoplamic Ca^{2+} ($[Ca^{2+}]i$) (A) and cytoplasmic pH (pHi) (B). Cultured porcine thyroid cells, loaded with fura-2 (A) or 2',7'-bis(2-carboxyethyl)-5 (and 6-) carboxyfluorescein (BCECF) (B), were stimulated with 100 ng/ml IGF-I at the point indicated by an arrow. Typical recordings (A1, B1) and the mean values (A2, B2) of $[Ca^{2+}]i$ and pHi are shown. Each point in A2, B2 is the mean±SE of 6-15 determinations from different experiments.

Fig. 2. Effect of graded doses of dimethylamiloride (DMA) on 100 ng/ml IGF-I-stimulated $[Ca^{2+}]i$ response (peak $[Ca^{2+}]i$ response) (A) and alkalinization ((dpHi/dt)max) (B). The maximal rate of changes of pHi/min ((dpHi/dt)max) (B) was obtained from the steepest slopes of original fluorescence tracings. Each point is the mean±SE of 6-15 determinations from different experiments.

occurred immediately with no detectable lag, reached a maximum within 2 minutes, and then slowly declined. The cells were loaded with BCECF and then pHi was recorded continuously. The resting pHi of porcine thyroid cells was 7.22±0.03 (n=15). Addition of 100 ng/ml IGF-I to these cells induced intracellular alkalinization, producing a pHi of 7.38±0.05 (n=15) in 2 min (Fig. 1B). Addition of IGF-I to thyroid cells leads to a rapid rise in $[Ca^{2+}]i$ and a simultaneous increase in pHi; under the conditions used, IGF-I causes an intracellular Ca^{2+}-mobilization and a concomitant activation of the Na^+/H^+-antiporter.

Dimethylamiloride (DMA) depresses the IGF-I-induced $[Ca^{2+}]i$ response and alkalinization (Fig. 2). Half maximal inhibition of the IGF-I-induced $[Ca^{2+}]i$ response is observed at approximately 35 μM DMA (Fig. 2A). This value is close



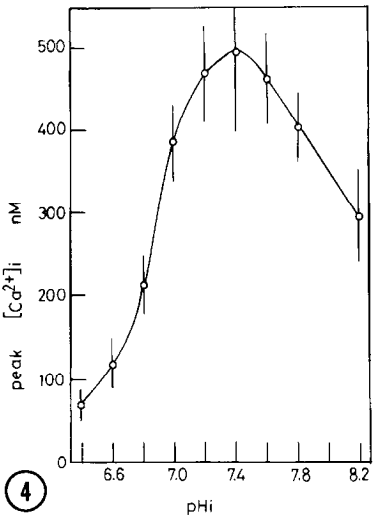


Fig. 3. Effects of extracellular Na^+ concentration ($[Na^+]o$) on 100 ng/ml IGF-I-stimulated increase in $[Ca^{2+}]i$ (A) and alkalinization ((dpHi/dt)max) (B). The maximal rate of changes of pHi/min (dpHi/dt)max) (B) was obtained from the steepest slopes of original fluorescence tracings. $[Na^+]o$ was lowered by isoosmotic replacement with choline$^+$. Each point is the mean±SE of 6-15 determinations from different experiments.

Fig. 4. Effect of cytoplasmic pHi on $[Ca^{2+}]$ response to 100 ng/ml IGF-I. The cells, loaded with fura-2, were kept in PBSG-HEPES containing 0.7 µg/ml nigericin at pH values as indicated for 5 min. The correct pHi values measured by a calibration procedure as described in the methods are the same as extracellular pH under these conditions. Subsequently the cells were stimulated with 100 ng/ml IGF-I. Each point is the mean±SE of 6-15 determinations from different experiments.

to the DMA concentration required for half maximal inhibition of the Na^+/H^+-antiporter (Fig. 2B).

To eliminate non-specific DMA effect, the Na^+/H^+-antiporter was inhibited by a decrease in the extracellular Na^+ ($[Na^+]o$). As shown in Fig. 3A, a reduction of $[Na^+]o$ leads to a depression of the $[Ca^{2+}]i$ response to IGF-I. The inhibition of the $[Ca^{2+}]i$ response occurs at the same Na^+-concentrations which leads to a decrease in alkalinization (Fig. 3B).

The data presented so far suggest a correlation between the Na^+/H^+-antiporter activity and $[Ca^{2+}]i$ response. The results do not permit, however, to decide whether the observed attenuation of the $[Ca^{2+}]i$ response is caused by an increase in cytoplasmic $[H^+]$ or a decrease in intracellular Na^+ ($[Na^+]i$). Therefore, the effect of various pHi-levels on the $[Ca^{2+}]i$ response to IGF-I was studied at a constant $[Na^+]o$-concentration. Fig. 4 demonstrates a pronounced pHi-optimum of the Ca^{2+}-mobilization around pHi 7.3-7.4. It is

evident from Fig. 4 that such an alteration in pHi enhances the intracellular Ca^{2+}-mobilization. Acidification depresses the $[Ca^{2+}]i$ response to IGF-I; the $[Ca^{2+}]i$ response to IGF-I is a pHi-dependent process which is drastically reduced at pHi-values below 6.8.

DISCUSSION

The $[Ca^{2+}]i$ response to IGF-I is a pHi-dependent process. IGF-I activates Na^+/H^+-antiporter and alkalinizes thyroid cells. The resulting increase in pHi facilitates the $[Ca^{2+}]i$ response by adjusting the pHi closer to the pHi-optimum of the intracellular Ca^{2+}-mobilizing system. Inhibition of the Na^+/H^+-antiporter by dimethylamiloride or a reduction of external $[Na^+]o$-concentrations attenuates the $[Ca^{2+}]i$ response to IGF-I. Thus one of the biological functions of the IGF-I-stimulated activation of the Na^+/H^+-antiporter is to shift the pHi to an optimal value for the $[Ca^{2+}]i$ response.

IGF-I stimulates the proliferation of primary cultured porcine thyroid cells (10). However, we do not know much about the intracellular pathways that mediate the action of IGF-I on thyroid cell proliferation. It has been suggested that stimulation of Na^+/H^+ exchange, producing a rapid and persistent alkalinization, is associated with mitogen action in vertebrate cells (11-16). Mitogens rapidly activate a Na^+/H^+ exchange mechanism in the plasma membrane of their target cells, implicating cytoplasmic pHi as a potential signal for cell proliferation. Grinstein et al. (16) listed several mitogens, which activate Na^+/H^+ exchange, including EGF (epidermal growth factor), PDGF (platelet derived growth factor), CSF (colony stimulating factor), and NGF (nerve growth factor). Activation with IGF-I had, however, not been presented until 1990, when IGF-I was reported to alkalinize porcine thyroid cells (1) and FRTL-5 cells (2). IGF-I, EGF, and phorbol ester stimulate porcine thyroid cell alkalinization and proliferation (1,8). Thus mitogen-stimulated alkalinization appears to be a ubiquitous phenomenon in the proliferation process of the primary cultured porcine thyroid cells. One of the early responses to IGF-I is thyroid cell alkalinization.

IGF-I is also known to induce an increase in $[Ca^{2+}]i$ (3), which represents the early cellular response to IGF-I in the thyroid gland; the $[Ca^{2+}]i$ response to IGF-I may be a signal for cell proliferation (3). A question, to which we have no answer as yet, is what is the interrelationship between the Na^+/H^+-antiporter and the intracellular $[Ca^{2+}]i$ response system in the thyroid cells. Thus we studied this interrelationship. We concluded that one of the biological functions of IGF-I-stimulated activation of the Na^+/H^+-antiporter is to shift the cytoplasmic pHi to an optimal value for the $[Ca^{2+}]i$ response. Interrelationship between the Na^+/H^+-antiporter and the intracellular Ca^{2+}-mobilization system is reported in platelets (17-19) and fibroblasts (20); the inhibitors of the Na^+/H^+-antiporter have been reported to inhibit the $[Ca^{2+}]i$ response in both cells. In the fibroblasts, the growth factor-induced activation of the Na^+/H^+-antiporter facilitates the Ca^{2+}-mobilization by shifting the pHi towards the optimum for the $[Ca^{2+}]i$ response, which is consonant with our observation in the thyroid cells.

IGF-I has been isolated from human plasma and plays an important role in cell growth. Patients with acromegaly always have elevated levels of plasma IGF-I. IGF-I may be involved in the formation of goiter in these patients. The data presented here indicate that intracellular Ca^{2+}-mobilization by IGF-I is a pHi-dependent process with a pHi-optimum around 7.2-7.4. Inhibition of the Na^+/H^+-antiporter attenuates the $[Ca^{2+}]i$ response to IGF-I. The activation of the Na^+/H^+-antiporter facilitates the Ca^{2+}-mobilization by shifting the pHi towards the optimum for the $[Ca^{2+}]i$ response.

REFERENCES

1. Takasu, N., Komiya, I., Nagasawa, Y., Asawa, Y., Shimizu, Y., and Yamada, T. (1990) J. Endocrinol. 127, 305-309.
2. Woods, D.J., Soden, J., Tomlinson, S., and Bidey, S.P. (1990) J. Mol. Endocrinol. 4, 177-185.
3. Takasu, N., Takasu, M., Komiya, I., Nagasawa, Y., Asawa, T., Shimizu, Y., and Yamada, T. (1989) J. Biol. Chem. 264, 18485-18488.
4. Takasu, N., Charrier, B., Mauchamp, J., and Lissitzky, S. (1978) Eur. J. Biochem. 90, 131-138.
5. Takasu, N., Handa, Y., Shimizu, Y., and Yamada, T. (1984) J. Endocrinol. 101, 189-196.

6. Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440-3450.
7. Rink, T.J., Tsien, R.Y., and Pozzan, T. (1982) J. Cell Biol. 95, 189-196.
8. Takasu, N., Komiya, I., Nagasawa, Y., Asawa, T., Shinoda, T., Yamada, T. and Shimizu, Y. (1990) Am. J. Phys. 258, E445-450.
9. Thomas, J.A., Buchsbaum, R.N., Zimniak, A., and Racker, E. (1979) Biochemistry 18, 2210-2218.
10. Saji, M., Tsushima, T., Isozaki, O., Murakami, H., Ohba, Y., Sato, K., Arai, M., and Shizume, K. (1987) Endocrifology 121, 749-756.
11. Rozengurt, E. (1981) Adv. Enzyme Regul. 19, 61-85.
12. Moolenaar, W.H., Yarden, Y., de Laat, S.W., and Schlessinger, J. (1982) J. Biol. Chem. 257, 8502-8506.
13. Poussegur, J. (1985) Trends Biochem. Sci. 10, 453-455.
14. Grinstein, S., and Rothstein, A. (1986) J. Membr. Biol. 90, 1-12.
15. Moolenaar, W.H. (1986) Annu. Rev. Physiol. 48, 363-376.
16. Grinstein, S., Rotin, D., and Mason, M.J. (1989) Biochim. Biophys. Acta 988, 73-97.
17. Siffert, W., and Akkerman, J.W.N. (1987) Nature 325, 456-458.
18. Ghigo, D., Treves, S., Turrini, F., Pannocchia, A., Pescarmona, G., and Bosia, A. (1989) Biochim. Biophys. Acta 940, 141-148.
19. Zavoico, G.B., Cragoe, Jr. E.J., and Feinstein, M.B. (1986) J. Biol. Chem. 261, 13160-13167.
20. Maly, K., Hochleitner, B.W., and Grunicke, H. (1990) Biochem. Biophys. Res. Commun. 167, 1206-1213.