Relationship between Proliferation and Cell Cycle-dependent Ca^{2+} Influx Induced by a Combination of Thyrotropin and Insulin-like Growth Factor-I in Rat Thyroid Cells

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Abstract

The mechanism of cell proliferation by a combination of thyroid-stimulating hormone (TSH) and insulin-like growth factor-I (IGF-I) was studied in rat thyroid (FRTL-5) cells. IGF-I stimulated an ~ 3.5-fold increase in the rate of Ca^{2+} influx sustained for at least 6 h in TSH-pretreated cells but not in quiescent cells. The significant cell proliferation was observed when TSH-primed cells were incubated with IGF-I for 24 h but not for 12 h. IGF-I stimulated the rate of Ca^{2+} influx in a dose-dependent manner that was similar to that for induction of DNA synthesis. Both Ca^{2+} influx and DNA synthesis observed in response to IGF-I in TSH-primed cells were inhibited by cobalt. In addition, the stimulations of Ca^{2+} influx and DNA synthesis by IGF-I were dependent on extracellular Ca^{2+} in TSH-pretreated cells. When TSH-primed cells were pretreated with pertussis toxin, both IGF-I-induced Ca^{2+} influx and DNA synthesis were abolished. However, pertussis toxin did not block the priming action of TSH or forskolin. When calcium entry was induced by Bay K8644, it stimulated cell growth in TSH-primed cells but not in quiescent cells. Moreover, cobalt and lanthanum inhibited DNA synthesis even when added several hours after the addition of Bay K8644 but not when added 24 h after the growth factor in TSH-primed cells. These findings suggest that at least two important mechanisms may work in response to IGF-I only in the TSH-primed G_1 phase of the cell cycle: first, IGF-I can activate directly or indirectly the Ca^{2+} channel via a pertussis toxin-sensitive substrate in TSH-primed cells; and second, a long lasting calcium entry by IGF-I may be a cell cycle-dependent mitogenic signal. (*J. Clin. Invest.* 1990. 86:1548–1555.) Key words: cell cycle • progression • Ca^{2+} influx • pertussis toxin • proliferation

Introduction

Proliferation of cells can be induced by combined addition of two or more growth factors. Competence factors render G_0-arrested quiescent cells to become competent and these cells are capable of progressing through G_1 to S phase of the cell cycle in response to progression factors (1–3). Thus, the actions of growth factors are dependent on the cell cycle. In rat thyroid (FRTL-5) cells, thyroid-stimulating hormone (TSH) may function at least as a competence factor and make cells competent to respond to a progression factor, insulin-like growth factor I (IGF-I),[1] which may enable the cells to progress through G_1 to S (4). Therefore, IGF-I markedly stimulates DNA synthesis in cells pretreated with TSH but not in G_0-arrested quiescent cells. It is, however, not clear how these growth factors acting on cell surface receptors promote the synergistic effect on mitogenesis. In this regard, events that occur in the G_1 stage of the cell cycle are commonly thought to regulate mitogenesis (5). Therefore, understanding the molecular events that occur during the G_1 stage after stimulation of G_0-arrested cells which made them proliferate, should provide important information about the mechanism of growth regulation. In this paper, the nature of the events that occur when cells move out of the G_0-arrested quiescent state into the active cell cycle was studied.

Most effects of TSH on the thyroid are mediated through activation of the adenylate cyclase-cyclic AMP system (6). Additionally, TSH is reported to stimulate phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis (7, 8). On the other hand, IGF-I activates the tyrosine kinase activity of the IGF-I receptors (9, 10). Moreover, the activations of these second messengers by IGF-I or TSH are not dependent on the cell cycle. However, the effects of IGF-I on mitogenesis are observed in TSH-pretreated cells but not in quiescent cells. Taken together, it seems possible that IGF-I binding of specific receptors might result in the generation of other mitogenic signals than the activation of tyrosine kinase in the G_1 phase of the cell cycle. Calcium ion and calmodulin, the major Ca^{2+} receptor which is the mediator of many Ca^{2+}-dependent intracellular processes, play important regulatory functions in the cell cycle regulation (11–13). When the concentration of extracellular free calcium is reduced, cells do not proliferate (14, 15). Based on these considerations, it is proposed that IGF-I may be capable of producing a calcium-related mitogenic signal in a cell cycle-dependent manner.

To examine this hypothesis, we have investigated the effect of IGF-I on the calcium influx in TSH-pretreated competent cells or quiescent cells. Furthermore, we have analyzed the relationship between calcium influx and mitogenesis induced by a combination of TSH and IGF-I with the use of pertussis toxin.

Methods

Materials. Bovine TSH, insulin, transferrin, somatostatin, hydrocortisone, glycyl-L-histidyl-L-lysine acetate, fatty acid free bovine serum albumin, verapamil and nitrendipine were purchased from Sigma

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Received for publication 4 December 1989 and in revised form 22 June 1990.

J. Clin. Invest.

0021-9738/90/11/1548/08 $2.00
Volume 86, November 1990, 1548–1555

1. *Abbreviations used in this paper:* DAG, diacylglycerol; IGF-I, insulin-like growth factor-I; PT, pertussis toxin.

Chemical Co. (St. Louis, MO); biosynthetic IGF-I from Amersham Corp. (Arlington Heights, IL); Coon's modified Ham's F-12 medium and calf serum from Gibco Laboratories (Grand Island, NY); pertussis toxin from Funakoshi Co. (Tokyo, Japan); Bay K8644 from Calbiochem-Behring Corp. (San Diego, CA); [^{45}Ca]$CaCl_2$ and [^{3}H]thymidine from DuPont (Wilmington, DE)-New England Nuclear (Boston, MA); other materials and culture medium were obtained from commercial sources.

Cell culture. FRTL-5 cells were maintained in Coon's modified Ham's F-12 medium supplemented with 5% calf serum and six hormones: TSH (0.3 mU/ml), insulin (10 μg/ml), transferrin (5 μg/ml), somatostatin (10 ng/ml), cortisone (10 nM), and the tripeptide glycyl-L-histidyl-L-lysine acetate (10 ng/ml) (6H medium) as described previously (16). Cells were incubated at 37°C in a humidified atmosphere (95 air, 5% CO_2) and the culture medium was changed twice weekly. In preparation for experiments, quiescent cells were obtained as described (17) with some modification. In short, FRTL-5 cells were seeded into 24-well or 12-well costar trays (~ 2×10^5 or 4×10^5 cells/well, respectively) and incubated for 3 d in 6H medium containing 5% calf serum. Cells were washed twice with PBS and then incubated for 2 d in basal medium consisting of Coon's modified Ham's F-12 medium and 0.25% BSA. For TSH treatment, quiescent cells were incubated for 5 h in Coon's modified Ham's F-12 medium containing 0.25% BSA and 0.1 mU/ml TSH, and then washed three times with PBS (TSH-primed cells).

Measurement of Ca^{2+} influx. Ca^{2+} influx was determined by measuring the ^{45}Ca uptake (18, 19). Quiescent or TSH-primed cells were obtained in a 12-well plate. Cells were incubated for the indicated time in Coon's modified Ham's F-12 medium containing 0.25% BSA and with or without one or more of the following compounds: IGF-I, cobalt, verapamil, nitrendipine, Bay K8644, and pertussis toxin. The assay was then started by changing the medium to the labeled F-12 medium containing 5 μCi/ml ^{45}Ca. Cells were incubated at 37°C for 30, 60, 90, or 120 s, and the reaction was terminated by aspirating the labeled medium. Cells were washed five times with ice-cold PBS containing 25 mM $MgCl_2$ to displace ^{45}Ca bound extracellulary and were lysed in 1 M NaOH, and the ^{45}Ca content of the lysate was determined by liquid scintillation spectrometer. The rate of Ca^{2+} influx was calculated by using a slope of the linear regression line of Ca^{2+} uptake.

Measurement of cytoplasmic Ca^{2+} in Fura-2-loaded cells. The cytoplasmic free calcium concentration was determined by measuring Fura-2 fluorescence as described (20, 21). Fura-2/AM was added to the cell suspension at a final concentration of 1 μM and incubated for 30 min at 37°C. Fluorescence was monitored in a fluorescence spectrophotometer with an excitation wavelength of 340 nm and an emission wavelength of 505 nm, using a cuvette maintained at 37°C. The cytoplasmic free Ca^{2+}, $[Ca^{2+}]_i$ was calculated by the equation: $[Ca^{2+}]_i = K_d \times (F - F_{min})/(F_{max} - F)$, using K_d = 224 nM accordingly (22).

Measurement of cell proliferation. Cell proliferation was assessed by the measurement of cell numbers as well as by [^{3}H]thymidine incorporation into cellular DNA. DNA synthesis was assessed by measuring [^{3}H]thymidine incorporation into trichloroacetic acid precipitable materials. Quiescent or TSH-primed cells were obtained in a 24-well plate as described above. Cells were then incubated for 43 h in RPMI 1640 medium containing 0.25% BSA and [^{3}H]thymidine (0.5 μCi/well) and with or without one or more of the following agents: IGF-I, Bay K8644, cobalt, verapamil, nitrendipine, lanthanum, and pertussis toxin. The reaction was stopped by addition of 10% TCA and the radioactivity in acid-insoluble materials was counted in a liquid scintillation spectrometer. Cell number was determined using a Coulter counter (Coulter Electronics Inc., Hialeah, FL) after removing cells from dishes with trypsin-EDTA solution at the indicated time.

Results

Effect of IGF-I on Ca^{2+} influx. Unstimulated TSH-primed cells showed a time-dependent accumulation of [^{45}Ca] and the rate of uptake of radioactivity into the cells was linear (r = 0.99) up to at least 120 s (Fig. 1). Ca^{2+} influx rate was calculated from the slope of the uptake line. When 1 nM IGF-I was added 30 min before the addition of [^{45}Ca], at time 0 TSH-primed cells accumulated more radioactivity than unstimulated cells at each time point. The uptake of [^{45}Ca] into IGF-I-added cells was also linear (r = 0.98) within the first 120 s. When 40 mM K^+ was added with [^{45}Ca] at time 0, the accumulation of radioactivity into the cells was greater than that in unstimulated cells and the uptake of [^{45}Ca] was linear (r = 0.96) up to 120 s.

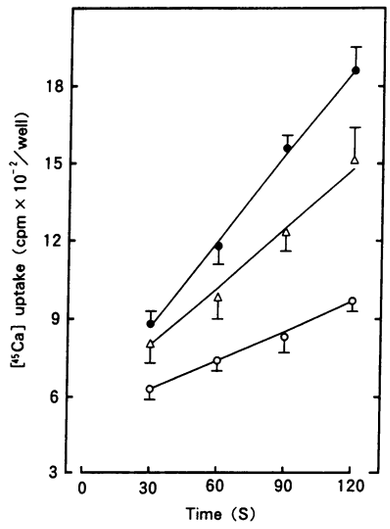

Figure 1. Uptake of [^{45}Ca] into TSH-primed cells. [^{45}Ca] and KCl were added at 0 s, and IGF-I was added 30 min before the addition of [^{45}Ca]. [^{45}Ca] uptake was measured at 30, 60, 90, and 120 s at 37°C as described under Methods. (— ○ —) Control; (— ● —) 1 nM IGF-I; (— △ —) 40 mM KCl. Points and bars represent the means±SE for three experiments each done in triplicate.

Addition of IGF-I to the cells pretreated for 5 h with TSH, forskolin, or Bt_2cAMP markedly produced increases in the rate of Ca^{2+} influx (Fig. 2 *A*). In contrast, IGF-I did not stimulate Ca^{2+} influx in quiescent cells when presented to the cells either alone or concomitantly with TSH or Bt_2cAMP (Fig. 2 *B*). In addition, IGF-I did not increase the Ca^{2+} influx rate in the cells preincubated for 0.5 or 1 h with TSH (data not shown).

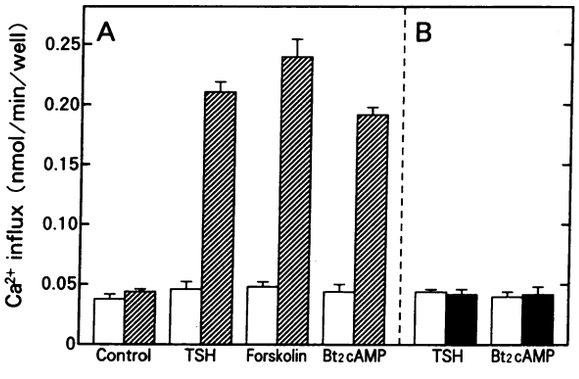

Figure 2. Effect of pretreatment with TSH, forskolin, or Bt_2cAMP on IGF-I-stimulated Ca^{2+} influx. (*A*) Quiescent cells were pretreated with 0.1 mU/ml TSH, 10 μM forskolin, or 0.1 mM Bt_2cAMP for 5 h and then washed three times with PBS. Following treatment, cells were incubated for 0.5 h with (*hatched column*) or without (*open column*) 1 nM IGF-I. (*B*) Quiescent cells were incubated for 0.5 h with (*closed column*) or without (*open column*) 1 nM IGF-I in the presence of 0.1 mU/ml TSH or 0.1 mM Bt_2cAMP, then [^{45}Ca] uptake was measured. Columns and bars in *A* and *B* indicate the means±SE for three experiments each done in triplicate.

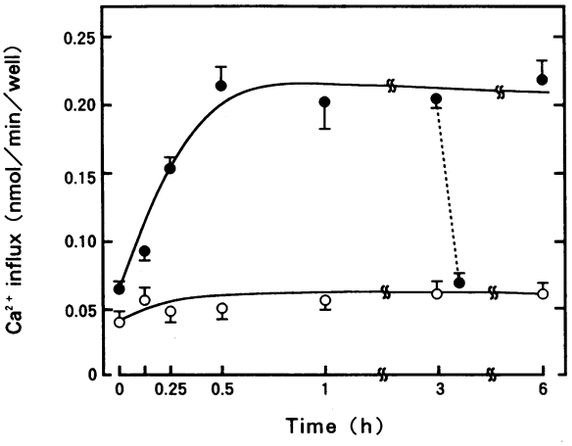

Figure 3. Time course for stimulation of Ca^{2+} influx by IGF-I. Quiescent cells (○) or TSH-primed cells (●) were incubated for the indicated time with 1 nM IGF-I. At 3 h, IGF-I was removed in TSH-primed cells (— ● —). Ca^{2+} influx rate was measured as described under Methods. Points and bars represent the means±SE for three experiments each done in triplicate.

When TSH-primed cells were incubated with 1 nM IGF-I, the rate of Ca^{2+} influx increased gradually (Fig. 3). At 30 min of the addition of IGF-I, the rate of Ca^{2+} influx was ~ 3.5-fold of the basal rate. The effect of IGF-I continued for at least 6 h. Within 3 min after the removal of IGF-I, the rate of Ca^{2+} influx decreased to the basal rate.

Quiescent or TSH-primed cells were loaded with Fura-2 and changes in cytoplasmic free Ca^{2+} concentration $[Ca^{2+}]_i$ were monitored. Basal $[Ca^{2+}]_i$ was 121±16 nM (mean±SE, n = 6) and the change in $[Ca^{2+}]_i$ induced by IGF-I was not observed in quiescent cells (Fig. 4 *A*). In TSH-primed cells, however, basal $[Ca^{2+}]_i$ was 131±25 nM (n = 6) and addition of IGF-I resulted in a gradual increase in $[Ca^{2+}]_i$ starting at ~ 12 min after the addition of IGF-I (Fig. 4 *B*). The IGF-I–induced $[Ca^{2+}]_i$ response reached a steady state level of 348±31 nM (n = 6) at ~ 30 min and remained to be elevated for at least an additional 150 min.

Thus, IGF-I induced both sustained increase in the rate of Ca^{2+} entry and prolonged elevation of cytoplasmic free Ca^{2+} concentration in TSH-primed cells but not in quiescent cells.

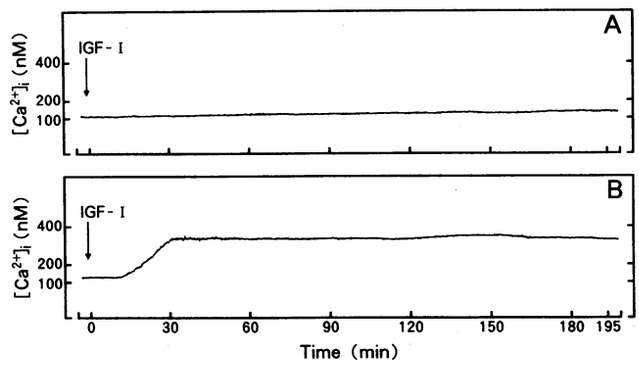

Figure 4. Effect of IGF-I on cytoplasmic free calcium concentration in FRTL-5 cells. Quiescent cells (*A*) or TSH-primed cells (*B*) were loaded with Fura-2. Cells were stimulated by 1 nM IGF-I at the point indicated by an arrow.

Table I. Effect of IGF-I Treatment on Cell Growth

Incubation time	[^{3}H]Thymidine incorporation	Cell number
h	*cpm × 10^{-3}/μg DNA*	*×10^{-5}/well*
Quiescent cells		
0	2.72±0.38	2.3±0.45
12	2.68±0.51	2.3±0.19
43	3.74±0.64	2.8±0.65
TSH-primed cells		
0	3.21±0.44	2.6±0.27
3	3.34±0.56	2.5±0.55
6	3.26±0.78	2.6±0.69
12	3.74±0.32	2.7±0.21
24	9.83±0.97	5.3±0.87
43	11.41±0.66	5.9±0.53

Quiescent or TSH-primed cells were incubated with 1 nM IGF-I for the indicated culture times. [^{3}H]Thymidine incorporation was measured at 43 h and cell number was determined at 67 h, respectively. When cells were incubated for 12 h with IGF-I and washed three times with PBS, [^{3}H] thymidine and cell number were measured 31 and 55 h after removal of IGF-I, respectively. Values are the means±SE for three experiments each done in triplicate.

Effect of IGF-I treatment on cell growth. We examined growth responses of FRTL-5 cells to IGF-I by measuring the cell number and [^{3}H]thymidine incorporation into DNA. As shown in Table I, the significant cell growth was observed when TSH-primed cells were incubated with IGF-I for 24 but not 12 h. Thus, TSH-primed cells should be continuously exposed to IGF-I to enter S phase in the cell cycle.

Concentration dependence for IGF-I-induced Ca^{2+} influx and DNA synthesis. IGF-I stimulated the rate of Ca^{2+} influx in a dose-dependent manner (Fig. 5 *A*). The maximal effect of IGF-I was detected at 1 nM. Fig. 5 *B* illustrates a dose-dependent relationship for IGF-I-induced DNA synthesis. DNA synthesis was saturated at 1 nM. Thus, IGF-I caused the elevation of Ca^{2+} influx rate in a dose-dependent manner that was simi-

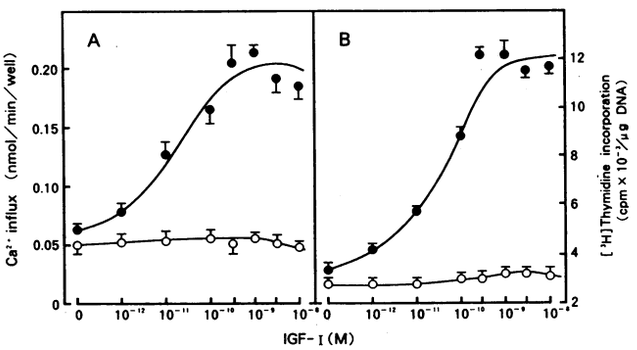

Figure 5. (*A*) Concentration dependence for Ca^{2+} influx by IGF-I. Quiescent cells (○) or TSH-primed cells (●) were incubated with various concentrations of IGF-I for 0.5 h, then the Ca^{2+} influx rate was measured. (*B*) Concentration dependence for DNA synthesis by IGF-I. Quiescent cells (○) or TSH-primed cells (●) were incubated for 43 h with the indicated concentration of IGF-I, then [^{3}H]thymidine incorporation was measured. Points and bars in *A* and *B* represent the means±SE for three experiments each done in triplicate.

lar to that for stimulation of DNA synthesis; half-maximal stimulation of both responses occurred with 5×10^{-11} M IGF-I.

Effects of Ca^{2+} channel blockers on IGF-I-induced Ca^{2+} influx and cell growth. A nonspecific inhibitor of the Ca^{2+} channel, cobalt inhibited both IGF-I-induced Ca^{2+} influx and DNA synthesis in a dose-dependent manner (Fig. 6). Cobalt completely blocked the action of IGF-I at 1 mM. The inhibitory action of cobalt may not be due to a nonspecific toxic effect since IGF-I stimulated both Ca^{2+} influx and DNA synthesis when extracellular Ca^{2+} concentration was raised to 8 mM. Moreover, 0.1 mM lanthanum also completely inhibited both Ca^{2+} influx and DNA synthesis induced by IGF-I (data not shown). Thus, when IGF-I-stimulated Ca^{2+} influx was pharmacologically blocked, IGF-I-induced DNA synthesis was also attenuated.

In order to characterize the type of Ca^{2+} channel responsible for an IGF-I-mediated increase in the rate of Ca^{2+} influx, we examined the effects of a number of Ca^{2+} channel antagonists on Ca^{2+} influx and cell growth by IGF-I. Inhibitors of voltage-dependent Ca^{2+} channels, such as verapamil or nitrendipine, neither reduced the rate of Ca^{2+} influx nor altered the cell growth stimulated by IGF-I (Table II). In contrast, verapamil and nitrendipine effectively inhibited the Ca^{2+} influx evoked by depolarization with 40 mM KCl.

Effects of extracellular Ca^{2+} on stimulation of Ca^{2+} influx and DNA synthesis by IGF-I. We next examined the effect of varying extracellular Ca^{2+} on IGF-I-stimulated responses. Extracellular Ca^{2+} stimulated weakly both the rate of Ca^{2+} influx and DNA synthesis in a dose-dependent manner in unstimulated TSH-primed cells. IGF-I stimulated Ca^{2+} influx approximately threefold at all extracellular Ca^{2+} concentrations. Therefore, IGF-I markedly stimulated the rate of Ca^{2+} influx in the presence of high concentrations of extracellular Ca^{2+} (Table III). A similar dependence on extracellular Ca^{2+} was found for DNA synthesis induced by IGF-I. Thus, the stimulations of Ca^{2+} influx and DNA synthesis observed in response to IGF-I were markedly dependent on extracellular Ca^{2+} in TSH-primed cells.

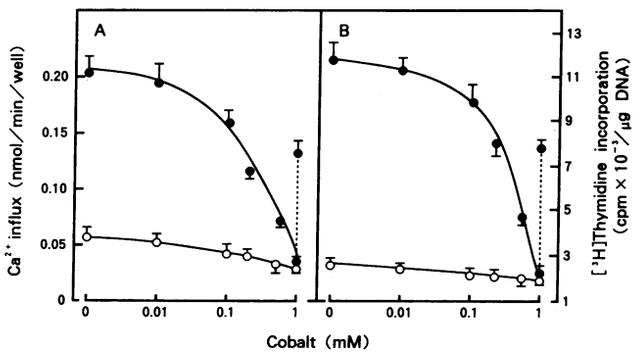

Figure 6. (*A*) Effect of cobalt on IGF-I-stimulated Ca^{2+} influx. TSH-primed cells were incubated for 0.5 h with (●) or without (○) 1 nM IGF-I in the presence of various concentrations of cobalt. At 1 mM cobalt, cells were stimulated by IGF-I in the presence of 8 mM extracellular Ca^{2+} (— ● —). (*B*) Effect of cobalt on IGF-I-induced DNA synthesis. TSH-primed cells were incubated for 43 h with (●) or without (○) 1 nM IGF-I in the presence of various concentrations of cobalt. At 1 mM cobalt, cells were incubated with IGF-I in the presence of 8 mM extracellular Ca^{2+} (— ● —). Points and bars in *A* and *B* represent the means±SE for three experiments each done in triplicate.

Table II. Effects of Ca^{2+} Channel Blockers on Ca^{2+} Influx and Cell Growth by IGF-I in TSH-primed Cells

Additions	Ca^{2+} influx	[^{3}H]Thymidine incorporation	Cell number
	pmol/min per well	*cpm × 10^{-3}/μg DNA*	*×10^{-5}/well*
None	60±2	2.71±0.09	2.1±0.30
Verapamil (50 μM)	51±9	2.53±0.35	1.9±0.32
Nitrendipine (1 μM)	62±3	2.70±0.13	2.0±0.16
IGF-I (1 nM)	221±24	13.8±1.03	6.7±0.85
+Verapamil (1 μM)	210±17	13.0±0.41	6.5±1.01
+Verapamil (10 μM)	212±20	13.1±0.67	6.5±0.83
+Verapamil (50 μM)	198±34	12.7±1.12	6.3±1.06
+Nitrendipine (0.1 μM)	220±13	13.3±0.74	6.5±0.55
+Nitrendipine (1 μM)	206±25	12.6±0.31	6.2±1.04
KCl (40 mM)	154±8	ND	ND
+Verapamil (10 μM)	59±12	ND	ND
+Nitrendipine (1 μM)	67±16	ND	ND

Cells were incubated with the indicated concentrations of verapamil or nitrendipine in the absence or presence of 1 nM IGF-I or 40 mM KCl for 0.5, 43, and 67 h for measurement of Ca^{2+} influx, [^{3}H]thymidine incorporation, and cell number, respectively. Values are the means±SE for three experiments each done in triplicate.

Effects of pertussis toxin on Ca^{2+} influx and DNA synthesis by IGF-I. We investigated the possibility of GTP-binding protein involvement in the action of IGF-I. Treatment of pertussis toxin at different times before and after addition of the growth factors showed that it inhibited DNA synthesis even when added several hours after TSH and IGF-I in quiescent cells (Fig. 7). Moreover, as shown in Table IV, addition of TSH plus

Table III. Effects of Extracellular Ca^{2+} on Stimulation of Ca^{2+} Influx and DNA Synthesis by IGF-I

Extracellular [Ca^{2+}]	Ca^{2+} influx	[^{3}H]Thymidine incorporation
mM	*pmol/min per well*	*cpm × 10^{-3}/μg DNA*
Without IGF-I		
0.1	34±7	1.81±0.32
0.5	54±8	2.83±0.48
1.0	72±12	3.40±0.25
2.0	82±7	3.86±0.41
4.0	88±6	4.12±0.46
With IGF-I (1 nM)		
0.1	84±9	3.91±0.51
0.5	150±22	10.0±1.14
1.0	194±11	13.2±0.64
2.0	248±24	17.0±0.78
4.0	261±16	17.6±0.65

TSH-primed cells were incubated with or without 1 nM IGF-I in the presence of various concentrations of extracellular Ca^{2+} for 0.5 and 43 h for measurement of Ca^{2+} influx and [^{3}H]thymidine incorporation, respectively. Values are the means±SE for three experiments each done in triplicate.

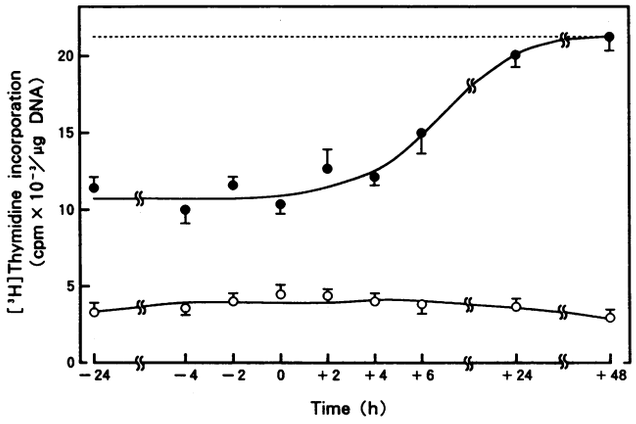

Figure 7. Inhibition of DNA synthesis in quiescent cells by pertussis toxin added at different times. Pertussis toxin (1 ng/ml) was added to the cells at the indicated times relative to the addition of 0.1 mU/ml TSH plus 1 nM IGF-I. [^{3}H]Thymidine incorporation was measured 48 h after the addition of the mitogens. +2 h represents addition of pertussis toxin 2 h after the growth factors and +48 h represents no addition of pertussis toxin. (●) With the mitogens; (○) without the mitogens. Points and bars represent the means±SE for three experiments each done in triplicate.

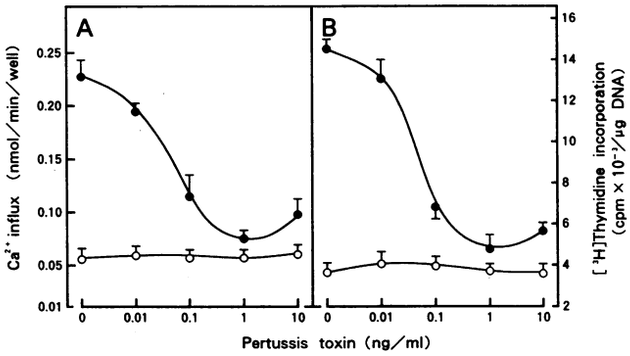

Figure 8. (*A*) Effect of pertussis toxin on IGF-I-stimulated Ca^{2+} influx. TSH-primed cells were treated with the indicated concentrations of pertussis toxin for 2 h and then were incubated for 0.5 h with (●) or without (○) 1 nM IGF-I. (*B*) Effect of pertussis toxin on IGF-I-induced DNA synthesis. TSH-primed cells were treated with the indicated concentrations of pertussis toxin for 2 h and then incubated for 43 h with (●) or without (○) 1 nM IGF-I. Points and bars in *A* and *B* represent the means±SE for three experiments each done in triplicate.

IGF-I to quiescent cells resulted in a gradual increase in the rate of Ca^{2+} influx starting at 2–4 h after the addition. At 8 h, a combination of TSH and IGF-I stimulated an ~ 3.5-fold increase in the rate of Ca^{2+} influx. Treatment of pertussis toxin also blocked the Ca^{2+} influx even when added 4–6 h after the addition of the growth factors. The time course of the inhibitory action of pertussis toxin on stimulated Ca^{2+} influx correlated well with that of the inhibitory action on DNA synthesis. Thus, pertussis toxin caused inhibition of the G_1 to S transition as well as attenuation of the Ca^{2+} influx by a combination of TSH and IGF-I in the cell cycle.

Pretreatment of TSH-primed cells with pertussis toxin inhibited the stimulation of Ca^{2+} influx by IGF-I in a dose-dependent manner (Fig. 8 *A*). Moreover, DNA synthesis induced by IGF-I was also inhibited in a dose-dependent manner by pretreatment with pertussis toxin (Fig. 8 *B*). At 1 ng/ml, pertussis toxin had a maximal effect. Therefore, doses of pertussis toxin that inhibited IGF-I-induced DNA synthesis correlated well with those to block IGF-I-stimulated Ca^{2+} influx. In contrast, IGF-I stimulated both Ca^{2+} influx and DNA synthesis when quiescent cells were primed with TSH or forskolin in the presence of pertussis toxin (Fig. 9). Thus, pertussis toxin did not block the priming action of TSH or forskolin.

Effect of Bay K8644 on Ca^{2+} influx and cell growth. We examined whether stimulation of Ca^{2+} influx resulted in an enhancement of cell growth with the use of Bay K8644, an agonist of voltage-dependent Ca^{2+} channel. As shown in Table V, Bay K8644 increased the rate of Ca^{2+} influx in FRTL-5 cells. The action of Bay K8644 was blocked by nitrendipine. When TSH-primed cells were incubated with Bay K8644, cell growth was markedly induced. In contrast, Bay K8644 did not induce cell proliferation in quiescent cells although it stimu-

Table IV. Effect of Pertussis Toxin on the Stimulation of Ca^{2+} Influx by TSH Plus IGF-I in Quiescent Cells

	Ca^{2+} influx (pmol/min per well)	
Time	Without pertussis toxin	With pertussis toxin
h		
−2	63±6	ND
0	62±9	64±10
+2	70±11	65±4
+4	159±18	98±26
+6	216±23	114±17
+8	228±19	168±24

TSH (0.1 mU/ml) and IGF-I (1 nM) were concomitantly added to quiescent cells at 0 h. Pertussis toxin (1 ng/ml) was added to cells at −2, 0, +2, +4, and +6 h, and the rate of Ca^{2+} influx was measured 2 h after the addition of pertussis toxin. Values are the means±SE for three experiments each done in triplicate.

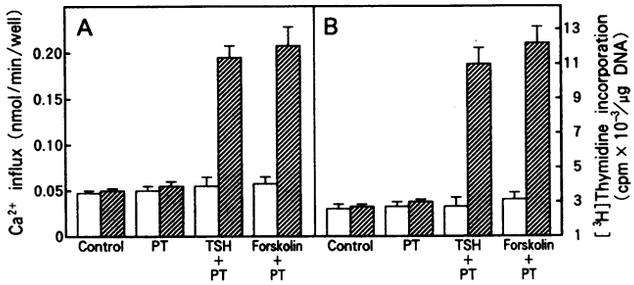

Figure 9. Effect of pertussis toxin on TSH or forskolin treatment in quiescent cells. Quiescent cells were preincubated with 1 ng/ml pertussis toxin (*PT*) for 12 h and treated with 0.1 mU/ml TSH or 10 μM forskolin for 5 h in the presence of PT. Cells were washed three times with PBS then further incubated for 3 h without PT and mitogens. Following the treatment, cells were incubated with (*hatched column*) or without (*open column*) 1 nM IGF-I for 0.5 h and 43 h for measurement of Ca^{2+} influx (*A*) and DNA synthesis (*B*), respectively. Columns and bars in *A* and *B* represent the means±SE for three experiments each done in triplicate.

Table V. Effects of Bay K8644 on Ca^{2+} Influx and Cell Growth in FRTL-5 Cells

Additions	Ca^{2+} influx	[^{3}H]Thymidine incorporation	Cell number
	pmol/min per well	*cpm × 10^{-3}/ μg DNA*	*×10^{-5}/well*
Quiescent cells			
None	52±4	2.06±0.17	1.8±0.07
Bay K8644 (1 μM)	121±16	2.55±0.62	2.0±0.18
TSH-primed cells			
None	65±8	2.98±0.40	2.1±0.32
Bay K8644 (1 μM)	128±12	8.60±0.93	4.7±0.54
+Nitrendipine (1 μM)	69±9	3.04±0.66	2.3±0.61
+Pertussis toxin (1 ng/ml)	132±17	8.72±0.81	4.7±0.91

Cells were incubated with or without 1 μM Bay K8644 in the absence or presence of 1 M nitrendipine for 0.5, 43, and 67 h for measurement of Ca^{2+} influx, [^{3}H]thymidine incorporation, and cell number, respectively. In some experiments, TSH-primed cells were pretreated with 1 ng/ml pertussis toxin for 2 h and incubated with Bay K8644. Values are the means±SE for three experiments each done in triplicate.

lated Ca^{2+} influx. Treatment of TSH-primed cells with pertussis toxin did not affect either Bay K8644-induced Ca^{2+} influx or cell proliferation. Furthermore, a number of Ca^{2+} channel antagonists, such as nitrendipine or lanthanum, inhibited DNA synthesis even when added several hours after the addition of Bay K8644 in TSH-primed cells (Fig. 10). Thus, a long lasting Ca^{2+} influx should be required for DNA synthesis in TSH-primed cells.

Discussion

Results obtained in this study clearly show that IGF-I stimulates sustained calcium entry in FRTL-5 cells preexposed to TSH, forskolin, or Bt_2cAMP. IGF-I-mediated Ca^{2+} influx remains for at least 6 h. The calcium entry decreases to the basal level after the removal of IGF-I. In addition, TSH-primed cells should be continuously exposed to IGF-I to enter S phase in the cell cycle. Based on these results, it is proposed that calcium entry stimulated by IGF-I may be a mitogenic signal in FRTL-5 cells pretreated with TSH. The following data support this idea: (*a*) IGF-I induces DNA synthesis when IGF-I stimulates Ca^{2+} influx; (*b*) reduction of IGF-I-stimulated Ca^{2+} influx by cobalt also attenuates IGF-I-induced DNA synthesis; (*c*) both IGF-I-induced Ca^{2+} influx and DNA synthesis are dependent on extracellular Ca^{2+} concentration; (*d*) treatment with pertussis toxin inhibits both IGF-I-stimulated Ca^{2+} influx and DNA synthesis; and (*e*) when Ca^{2+} influx is pharmacologically enhanced and remains for several hours in TSH-pretreated cells, DNA synthesis is induced.

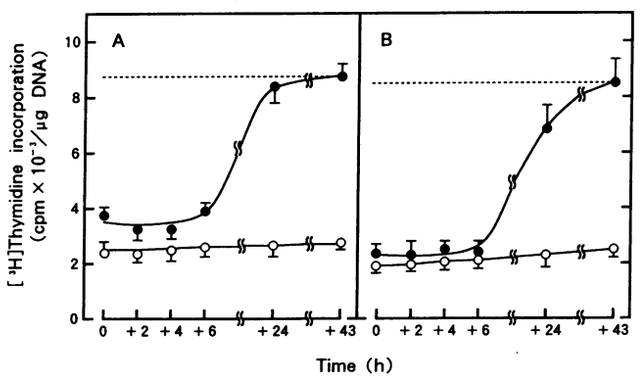

Figure 10. (*A*) Inhibition of Bay K8644-induced DNA synthesis by nitrendipine added at different times. Nitrendipine (1 μM) was added to TSH-primed cells at the indicated times relative to the addition of 1 μM Bay K8644. (●) With Bay K8644; (○) without Bay K8644. [^{3}H]Thymidine incorporation was measured 43 h after the addition of the mitogen. +43 h represents no addition of nitrendipine. (*B*) Inhibition of Bay K8644-induced DNA synthesis by lanthanum added at different times. Lanthanum (50 μM) was added at the indicated times relative to the addition of 1 μM Bay K8644. (●) With Bay K8644; (○) without Bay K8644. Points and bars in *A* and *B* represent the means±SE for three experiments each done in triplicate.

IGF-I is not capable of activating Ca^{2+} influx in quiescent cells. In addition, proliferation is not stimulated when Ca^{2+} influx is pharmacologically induced in quiescent cells. Thus, the effects of calcium entry and IGF-I on mitogenesis and Ca^{2+} influx, respectively, are dependent on the cell cycle. Therefore, TSH-treatment may induce dual critical changes in cells undergoing G_0 to G_1 transition. IGF-I can regulate the rate of Ca^{2+} influx and mitogenesis in cells pretreated with TSH for several hours. In this regard, the TSH-induced competent state may be achieved through a mechanism that involves, at least in part, the expression of specific competence genes. The c-*fos* and c-*myc* protooncogenes are contained within the competence gene family (23–25), and TSH stimulates the expression of these protooncogenes in FRTL-5 cells (26–28). Conceivably, part of the mitogenic response to TSH may appear to be mediated through the expression of c-*fos* and c-*myc*.

Pertussis toxin inhibited IGF-I-induced Ca^{2+} influx and mitogenesis in TSH-treated competent cells. However, pertussis toxin did not block the priming action of TSH. Further, pertussis toxin did not attenuate Bay K8644-stimulated Ca^{2+} influx and DNA synthesis in TSH-primed cells. These data demonstrate that pertussis toxin may block the action of IGF-I on cell cycle regulation. A high concentration of KCl as well as Bay K8644, an agonist of voltage-dependent Ca^{2+} channel, could stimulate calcium entry in FRTL-5 cells. In addition, the effects of these compounds were blocked by nitrendipine. In dog thyroid cells, 40 mM KCl does not elicit any change in cytoplasmic free calcium concentration (29). In contrast, TSH is capable of depolarizing cell membrane potentials (30) and Bay K8644 induces a rise in cytoplasmic free calcium (31) in cultured porcine thyroid cells. These data suggest that the effects of KCl, Bay K8644, and nitrendipine might not be nonspecific and that FRTL-5 cells would be electrically active. Inhibitors of voltage-sensitive Ca^{2+} channels did not abolish the Ca^{2+} influx induced by IGF-I. Therefore, IGF-I may regulate directly or indirectly the voltage-independent Ca^{2+} channel via a pertussis toxin-sensitive substrate, possibly GTP-binding protein. There are several reports of pertussis toxin inhibition of signaling by tyrosine kinase-containing receptors. Pertussis toxin inhibits mitogenesis induced by insulin or epidermal growth factor (32, 33).

Some growth factors stimulate the hydrolysis of phospholipids and the formation of second-messengers such as diacylglycerol (DAG) or inositol phosphates. Moreover, calcium entry is reported to be induced by second messengers gener-

ated following receptor activation (34, 35). In contrast, Ca^{2+} influx enhances the sustained production of DAG in leukocytes (36). IGF-I and insulin stimulate a phospholipase C which hydrolyses a glycosyl-phosphatidylinositol and the consequent formation of two second messengers, DAG and inositolphosphate-glycan (37, 38). Additionally, TSH plus IGF-I and TSH plus insulin synergistically elevate DAG content of FRTL-5 cells (39). Whether Ca^{2+} influx and DAG production by IGF-I plus TSH are parallel or sequential responses remains to be clarified.

On the other hand, it has been proposed that DAG induced by TSH plus insulin may activate protein kinase C, which may mediate the proliferative response in FRTL-5 cells (39). However, a combination of TSH and insulin can induce mitogenesis even in FRTL-5 cells in which the level of PKC is reduced (40). Moreover, PKC has a stimulatory effect on the G_0 to G_1 transition and an inhibitory effect on the G_1 to S transition in FRTL-5 cells (40). The role of DAG induced by IGF-I plus TSH in mediating the proliferative responses in FRTL-5 cells is not clear at present.

In summary, at least two important mechanisms may work in response to IGF-I only in TSH-primed G_1 phase of the cell cycle: first, IGF-I can activate directly or indirectly the Ca^{2+} channel via a pertussis toxin-sensitive substrate in TSH-primed cells; and second, a long lasting calcium entry by IGF-I may be a cell cycle-dependent mitogenic signal.

Acknowledgments

The authors are grateful to Miss Yuki Hirano for her secretarial assistance.

This work was supported by a research grant from the Intractable Disease Division, Public Health Bureau, Ministry of Health and Welfare, and Grant-in-Aid for Scientific Research (to Dr. Amino; No. 61480178) from the Ministry of Education, Science, and Culture of Japan.

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