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# Association of the Changes in Cytosolic $Ca^{2+}$ and Iodide Efflux Induced by Thyrotropin and by the Stimulation of $\alpha_1$-Adrenergic Receptors in Cultured Rat Thyroid Cells*

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**Thyrotropin causes a time- and concentration-dependent increase in cytosolic $Ca^{2+}$ in FRTL-5 rat thyroid cells as measured by Quin2 fluorescence; the half-maximal response occurs in response to $1 \times 10^{-7}$ M thyrotropin. The effect of added thyrotropin is the same whether cells have been previously and chronically exposed to thyrotropin or whether they have been thyrotropin "starved" for several days. The thyrotropin effect on cytosolic $Ca^{2+}$ has no relationship to intracellular cAMP levels with respect to dose and time course. Norepinephrine ($1 \times 10^{-7}$ M) also causes increases in cytosolic $Ca^{2+}$ in FRTL-5 thyroid cells. With the use of a variety of adrenergic inhibitors, norepinephrine was found to exert its effect via an $\alpha_1$-adrenergic receptor. The exposure of FRTL-5 cells to physiological thyrotropin concentrations enhances the effect on cytosolic $Ca^{2+}$ level induced by norepinephrine *in vitro*; the shape of the dose-response curve indicates a cooperative effect of the thyrotropin and norepinephrine. The increase in cytosolic $Ca^{2+}$ seems to be derived from an intracellular pool rather than from the extracellular space. It is not prevented by nifedipine, a blocker of $Ca^{2+}$ channels; it is present in cells exposed to ethylene glycol bis($\beta$-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; and it is not associated with increased $Ca^{2+}$ uptake into the cell. the thyrotropin- and norepinephrine-induced increase in cytosolic $Ca^{2+}$ parallels the efflux of iodide and the organification of thyroglobulin in a dose-dependent manner.**

Receptor-mediated changes in intracellular $Ca^{2+}$ have been shown to regulate cell functions in many biological systems (1–6). Although the regulatory action of $Ca^{2+}$ as a second messenger can be independent of the second messenger activity of cAMP (1–6), the two systems can be used to achieve a coordinate response greater than the sum of the parts (1–5). Thyrotropin (TSH[1]) stimulation of the iodination of thyroglobulin by the thyroid cell may be an example.

The iodination of thyroglobulin precedes the formation of thyroid hormone and requires a complex sequence of TSH-regulated events including iodide transport (7). In a continuous functioning line of cultured rat thyroid cells (FRTL-5), this sequence has been separated into TSH-stimulated events which are cAMP-mediated and non-cAMP-mediated (8–10). The cAMP-mediated process involves a sodium-dependent influx of iodide wherein the cell takes up iodide from the media and concentrates it (9). The non-cAMP-mediated TSH-stimulated process involves enhanced efflux from a thyroid cell of previously concentrated iodide (10). The efflux process cannot be stimulated by dibutyryl-cAMP (10) as could the influx event (9). It is evoked by norepinephrine, A23187, and arachidonic acid (10). A23187 is a $Ca^{2+}$ ionophore (11); arachidonic acid is an intermediate of $Ca^{2+}$-regulated turnover of phospholipids (12); and norepinephrine-stimulated $\alpha_1$-adrenergic receptors are coupled to intracellular $Ca^{2+}$ mobilization (13). The presence of EGTA in the medium blocks the norepinephrine and A23187 but not the arachidonic acid perturbation of iodide efflux (10). These observations suggested (10) that the TSH effect on iodide efflux might be associated with an increase in intracellular $Ca^{2+}$.

In the present report, we have directly evaluated the validity of this hypothesis by using the fluorescent indicator of cytosolic $Ca^{2+}$ levels, Quin2. This dye is characterized by a high affinity and specificity for $Ca^{2+}$, rapid and reversible kinetics, and large optical response to $Ca^{2+}$ binding (14–17). Quin2 has been used to evaluate the cytosolic free $Ca^{2+}$ in response to biological stimuli (15–17). In this report, we show that TSH, as well as norepinephrine via an $\alpha_1$-adrenergic receptor, caused a dose-dependent increase in FRTL-5 thyroid cell cytosolic $Ca^{2+}$ which paralleled the efflux of iodide. The increase in cytosolic $Ca^{2+}$ appears to be derived from intracellular $Ca^{2+}$ stores.

## EXPERIMENTAL PROCEDURES

*Cells*—The FRTL-5 cells used in this study are a continuous line of functional epithelial cells from rat thyroid. The isolation, growth, and basic characteristics of FRTL-5 cells have been described previously (18–20). The cells were grown in Coon's modified Ham's F-12 medium supplemented with 5% calf serum (Grand Island Biological Co., Grand Island, NY), 1 mM nonessential amino acids (Microbiological Associates, Bethesda, MD), and a 6-hormone (6H) mixture of TSH (100 microunits/ml), insulin (10 $\mu$g/ml), cortisol ($10^{-8}$ M), transferrin (5 $\mu$g/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml). Cells were passaged using a trypsin-collagenase mixture (18, 19). The cells were suspended in medium containing 2 mM EGTA for 5–10 min and vigorously aspirated through a pipette several times to ensure the separation of individual cells in suspension before seeding at a density of $2 \times 10^5$ cells/ml in 24-well or 10-cm tissue culture dishes (Costar, Cambridge, MA). This procedure resulted in a uniform dispersal of cells that grew as indi-

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[1] The abbreviations used are: TSH, thyrotropin; NE, norepinephrine; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HBSS, Hanks' balanced salt solution; Hepes, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

vidual cells in monolayer. Cells were grown in a 5% $CO_2$, 95% air (37 °C) atmosphere; the culture medium was changed every 3–4 days, and the cells were used in individual experiments 10–16 days after plating. Cells used in individual experiments had a media change 1 day earlier. Cells were maintained as noted either in the TSH-containing medium described above (6H cells) or in the same medium without TSH (5H cells); the duration of exposure to medium without TSH before an individual experiment was 5 days unless otherwise noted in the experimental protocol.

*Cell Loading with Quin2*—Suspensions of FRTL-5 cells were prepared as follows. 4–6 tissue culture dishes, each containing about $2 \times 10^7$ FRTL-5 cells, were washed with Hanks' balanced salt solution (HBSS); $Ca^{2+}$,$Mg^{2+}$-free HBSS containing 4 mM EGTA was then added. After incubation for 15–20 min in a shaking bath at 37 °C, complete detachment of the cells was achieved by pipetting the suspension several times. The cells were then collected by centrifugation at $500 \times g$ and washed once in HBSS. They were then resuspended in 10 mM Hepes-buffered (pH 7.3) growth medium containing TSH (6H) or without TSH (5H). Cells were maintained with slow shaking at 37 °C for 1 h. Cells were then centrifuged at $500 \times g$ and resuspended in Coon's modified F-12 medium containing 0.1% BSA. The fluorescent probe, Quin2 acetomethoxy ester, was added to the cell suspension at a final concentration of 10 μM. The incubation with Quin2 was carried out at 37 °C, under gentle shaking for 20 min. Cells were again centrifuged and resuspended in the Coon's medium containing 0.1% BSA. Aliquots were obtained from such suspension so as to have $1–2 \times 10^6$ cells/sample. They were kept at room temperature until needed. Under these conditions, no marked loss of Quin2 or decrease of cell viability occurred for several hours.

The intracellular Quin2 concentration was determined by evaluating the amount of Quin2 associated with the cells and the cell volume. The first figure was obtained by measuring the Quin2 fluorescence in disrupted cell suspensions and relating this value to a standard curve of Quin2 fluorescence. The cells' volume was obtained as previously described (8) and was 0.08 μl/μg DNA. The Quin2 intracellular concentration ranged from 1 to 3 mM in all the experiments.

*Determination of Cytosolic $Ca^{2+}$*—The labeled cells were washed in HBSS containing 0.02% BSA before each measurement and resuspended in 2 ml of the same salt solution ($\sim 1 \times 10^6$ cells/ml), at 37 °C. All fluorescence recordings were performed by a Perkin Elmer fluorescence spectrophotometer, model MPF-44A. The cuvette holder in this instrument was equipped with a built-in magnetic stirrer, and a double-jacketed wall through which constant temperature water could be circulated. Fluorescence signal was monitored as a function of time in cell suspensions kept under moderate stirring at 37 °C, with excitation at 339 nm and emission at 492 nm. The cell labeling was monitored in each sample by recording the fluorescence emission spectrum. The fluorescence signal of unloaded cells in suspension was negligible at the wavelengths where the Quin2 emission was measured.

The concentration of cytosolic $Ca^{2+}$ could be calculated from the fluorescence emission value ($F$) at 492 nm according to the following equation (16).

$$[Ca^{2+}] = 115 \text{ nM} \times \frac{(F - F_{min})}{(F_{max} - F)}$$

$F_{max}$ is the fluorescence intensity value at 492 nm upon disruption of the cells by sonication, and $F_{min}$ is the fluorescence signal at 492 nm after addition of 40 mM EGTA. Values were expressed either as absolute concentration of cytosolic $Ca^{2+}$ or as relative changes of such a concentration.

The viability of the cells before and after Quin2 loading and at the end of representative experiments was 97% as measured by trypan blue exclusion.

*cAMP Assay*—The intracellular cAMP content of FRTL-5 thyroid cells was measured by a modification of the method previously reported (21). In brief, 5H or 6H cells were washed twice with HBSS, and TSH at various concentrations ($1 \times 10^{-12}$ to $1 \times 10^{-6}$ M) in HBSS containing 0.4% BSA was added. Incubations were continued, unless otherwise noted, for 1 h at 37 °C in the presence of 0.5 mM 3-isobutyl-1-methylxanthine. The reaction was terminated by medium aspiration, the addition of 1 ml of cold absolute ethanol, and freezing overnight at −20 °C. Aliquots of the alcohol supernatant were dried and reconstituted with assay buffer (0.05 M sodium acetate (pH 6.2)). cAMP was measured using a radioimmunoassay (Becton Dickinson, Rutherford, NJ). DNA content was measured in the cell debris remaining on the plate after aspirating the alcohol supernatant (22). Results were usually expressed as picomoles/μg DNA. Experiments were performed in duplicate, as were the cAMP measurements in each well.

*Measurements of Steady-state Iodide Levels*—Iodide levels were measured in cells grown in 24-well plates (Costar, Cambridge, MA) by a procedure previously described (8–10), with the exception that incubations at 37 °C were terminated after 60 min, *i.e.* when steady-state conditions were achieved (10). Iodide levels were measured in parallel incubations performed in the presence of 10 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone which prevents iodide uptake (8–10). This value, which represents iodide in its diffusion space in the cells, was subtracted from the values presented. All experimental data were performed in triplicate at least twice. Results are reported as the mean ± S.E.

*Materials*—TSH was a purified preparation (23, 24), homogeneous in the ultracentrifuge ($M_r \sim 27.500$), with an activity of 26 ± 3 units/mg in the McKenzie assay (25). Crude TSH used in the tissue culture medium was obtained from Armour (Chicago, IL) and had a potency of about 0.1–0.3 IU/mg protein. Norepinephrine, propranolol, nifedipine, yohimbine, and EGTA ware purchased from Sigma. Phentolamine was obtained from Ciba Geigy (Summit, NJ). Prazosin was purchased from Pfizer. Quin2 and Quin2 methoxy ester were purchased from Calbiochem-Behring. All other chemicals were obtained from commercial sources as the highest purity material available.

## RESULTS

*Effects of TSH on Cytosolic $Ca^{2+}$ and on cAMP Levels*—In 17 different preparations, the cytosolic $Ca^{2+}$ concentration was calculated to be 115 ± 20 nM in 6H cells and 110 ± 15 nM in 5H cells. 6H cells are those chronically exposed to $\sim 1.4 \times 10^{-10}$ M TSH; 5H cells are those whose media has been depleted of TSH for at least 5 days (see "Experimental Procedures").

The addition of additional aliquots of TSH to either cell suspension resulted in an increase in the levels of cytosolic $Ca^{2+}$. For example, the effect of TSH as a function of time in 5H and 6H cells is shown in Fig. 1. The time dependence of the TSH effect was virtually identical in both 5H and 6H cells. A maximal effect was obtained within 3 min after the addition of TSH, followed by a small decrease (about 20% of the previous increase) that leveled off at 6 min (see Fig. 1) to a steady-state which was maintained for at least 20 min (not shown).

A TSH dose-response curve, obtained with both 6H and

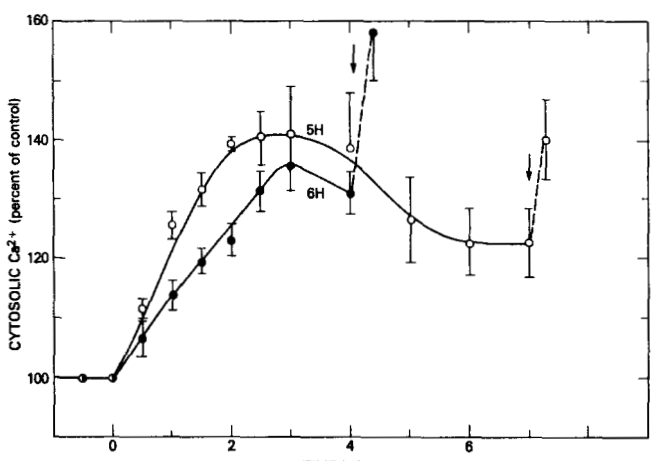


FIG. 1. **Time course of the variation in cytosolic $Ca^{2+}$ concentrations upon addition of $1 \times 10^{-7}$ M TSH to FRTL-5 cells in suspension.** The $Ca^{2+}$ level changes, expressed as per cent of the value at zero time, were followed in 5H (○) and 6H (●) cells. The *arrows* indicate the addition of $1 \times 10^{-5}$ M NE. The results are the mean of five determinations ± S.E. Similar results were obtained in experiments carried out with three different 6H or 5H cell preparations.

5H cell suspension, is shown in Fig. 2. Both cells responded to TSH stimulation in an identical manner with a half-maximal concentration of $1 \times 10^{-7}$ M (Fig. 2). These results are very different from the effect of TSH on the adenylate cyclase system of FRTL-5 cells. Thus, in the 5H cells, TSH induced a maximal increase of intracellular cAMP levels at $1 \times 10^{-10}$ M (Fig. 2). 6H cells by definition already have a TSH-stimulated cyclase with elevated steady-state cAMP levels due to the presence of the hormone in the medium. The small elevation of cAMP after the addition of TSH at $1 \times 10^{-6}$ M does not exceed levels seen in 5H cells; the minimal response is consistent with the well-recognized "desensitization" of the thyroidal adenylate cyclase response with chronic TSH treatment. The initially higher cAMP levels exhibited by $1 \times 10^{-10}$ M TSH in 5H by comparison to 6H cells (≅14 *versus* ≅6 pmol of cAMP/μg of DNA) is an overshoot phenomenon; within 1 day of TSH exposure, the cAMP level of the 5H cells falls to that of the 6H cells. It is apparent from the results shown in Fig. 2 that the effects of TSH on cAMP and on cytosolic $Ca^{2+}$ are dissociated in both 5H and 6H cells.

*Effect of Norepinephrine on Cytosolic $Ca^{2+}$*—Norepinephrine (NE) has been shown to have an effect on the iodide efflux in FRTL-5 cells similar to that of TSH (10). NE added to FRTL-5 cells in suspension caused an increase in the level of cytosolic $Ca^{2+}$. The time course of the NE effect in FRTL-5 cells is shown in Figs. 3 and 4, for 6H and 5H cells, respectively. The maximal increase in cytosolic $Ca^{2+}$ level was obtained 1 min after the addition of $1 \times 10^{-5}$ M NE in 6H cells (Fig. 3) and 5H cells (Fig. 4). This increase persists for about 60 s, then declines and levels off to a steady-state after 5–7 min. Fig. 5 shows the NE dose-response curve for cytosolic $Ca^{2+}$ level in 6H and 5H cells. The half-maximal dose was in both cases ~$5 \times 10^{-8}$ M NE. The amplitude of the response is greater in cells kept in 6H, and the pattern of the dose-response curve is different. This may relate to the polyphosphoinositide content which differs in 5H and 6H cells (30) and/or to a cooperative effect between TSH and NE. NE has no effect on the adenylate cyclase activity of 5H and 6H FRTL-5 thyroid cells as measured by cAMP levels (Table I).

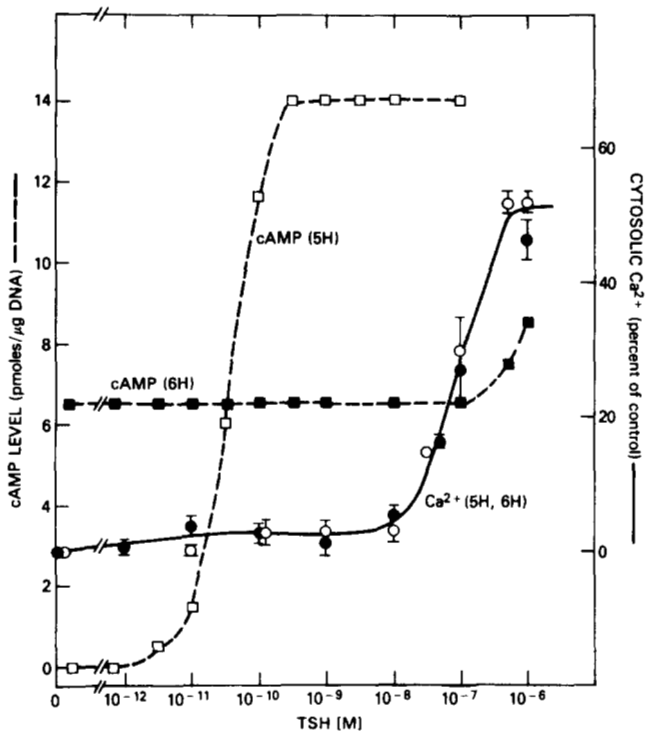


FIG. 2. **The effect of TSH on cytosolic $Ca^{2+}$ concentration and intracellular cAMP levels compared in 5H and 6H FRTL-5 rat thyroid cells.** The $Ca^{2+}$ concentration in FRTL-5 cells was evaluated from the Quin2 fluorescence signal, as described under "Experimental Procedures." The cell suspension was stirred, at 37 °C, during measurements. Additions of TSH were done in order to have the final concentrations indicated during the measuring period. Measurements were taken 3 min after each TSH addition. The cells used in the experiments were either 5H (○, □) or 6H (●, ■). The $Ca^{2+}$ levels are expressed as per cent of control (initial concentration in the absence of TSH). The results are the mean of four determinations ± S.E.; comparable results were obtained with >5 different cell preparations. The cAMP level were evaluated 5 min after each TSH addition, according to the procedure described under "Experimental Procedures."

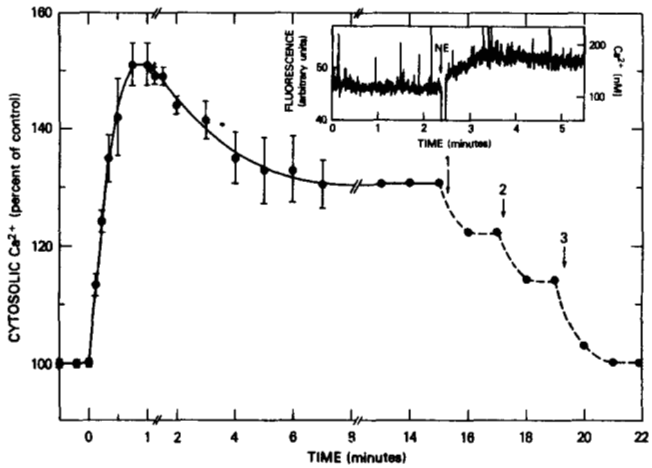


FIG. 3. **Time course of the changes in cytosolic $Ca^{2+}$ level in 6H cells upon addition of $1 \times 10^{-5}$ M NE.** The results, expressed as per cent of the value at the zero time, are the mean of three determinations ± S.E. Similar results were obtained with three different cell preparations. A representative experiment on the effect of α-adrenergic inhibitors on the $Ca^{2+}$ level is also shown. *Arrow 1* indicates the addition of $1 \times 10^{-8}$ M prazosin to the cell suspension and the subsequent decrease in the $Ca^{2+}$ level. *Arrows 2* and *3* indicate the addition of $3 \times 10^{-8}$ M prazosin and $1 \times 10^{-5}$ M phentolamine, respectively. As shown, the $Ca^{2+}$ level is brought back to the initial level, following the addition of α-adrenergic inhibitors. In the *inset*, a trace from the fluorometer shows the fluorescence emission level of 6H cells suspension before and after the addition of $1 \times 10^{-5}$ M NE. The corespondent $Ca^{2+}$ concentrations are also indicated.

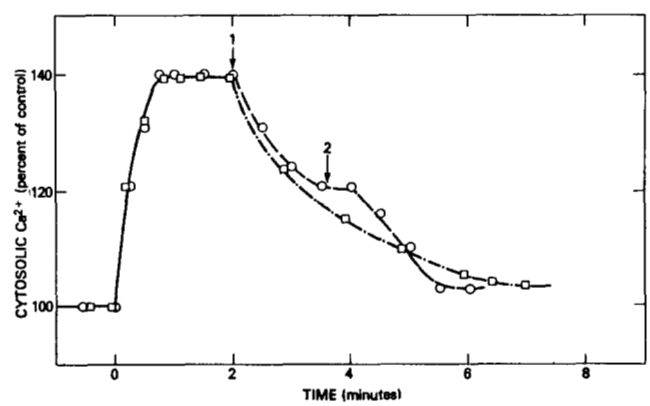


FIG. 4. **Time course of cytosolic $Ca^{2+}$ changes upon addition of $1 \times 10^{-5}$ M NE to 5H cells.** The effect of α blockers on the NE-induced $Ca^{2+}$ increase is also analyzed. *Arrow 1* indicates the addition of $3 \times 10^{-8}$ M prazosin (○—○) or $1 \times 10^{-4}$ M phentolamine (□- - -□). *Arrow 2* is the addition of $1 \times 10^{-5}$ M phentolamine (○—○). As shown the $Ca^{2+}$ value is brought back to the initial value by the α-adrenergic blockers. The experimental protocols are the same as in Fig. 3. Similar results were obtained with cells from three different preparations.

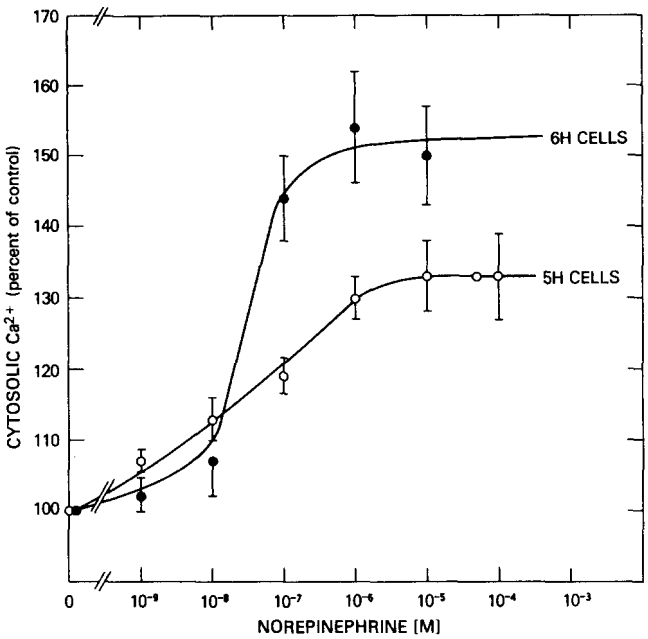


FIG. 5. **The effect of NE on cytosolic $Ca^{2+}$ concentration in FRTL-5 cells (5H, ○; 6H, ●).** Increasing concentrations of NE were added to the stirred cell suspensions kept at 37 °C. Measurements were taken 1 min after NE addition. The values are expressed as per cent of the $Ca^{2+}$ level in the absence of the ligand. The results are the mean of three different cell preparations and four separate determinations ± S.E.

TABLE I

*Effect of norepinephrine on the cAMP level of FRTL-5 thyroid cells*

Assays performed as described in Materials and Methods. Cells washed as per the multiple washes of the Quin2 loading procedure yielded the same results as did suspensions of cells loaded with Quin2.

| Ligand added | cAMP level | |
|---|---|---|
| | 5H FRTL-5 cells[a] | 6H FRTL-5 cells[a] |
| | *pmol/μg DNA* | |
| None | 0.2 | 6.4 |
| Norepinephrine | 0.2 | 6.2 |
| $1 \times 10^{-8}$ M | 0.2 | 6.2 |
| $1 \times 10^{-7}$ M | 0.3 | 6.6 |
| $1 \times 10^{-6}$ M | 0.2 | 5.8 |
| $1 \times 10^{-4}$ M | 0.2 | 6.0 |
| $1 \times 10^{-3}$ M | 0.3 | 6.4 |
| TSH ($1 \times 10^{-9}$ M) | 12.6 | |

[a] 6H cells are maintained in the presence of TSH throughout their lifespan prior to assay. 5H cells were changed to media free of added TSH 5 days before assay.

*Additivity of TSH and NE Effects*—The effects of TSH and NE on the cytosolic $Ca^{2+}$ level in FRTL-5 cells were shown to be additive. The addition of $1 \times 10^{-5}$ M NE, either at the point of the maximal TSH effect or at the steady-state (about 7 min after the TSH addition), resulted in a further increase of the cytosolic $Ca^{2+}$ level of about 30% (see Fig. 1). The same result was obtained when NE was added after a maximal dose of TSH ($1 \times 10^{-6}$ M) (data not shown). Conversely, when $1 \times 10^{-7}$ M TSH was added to 6H cells stimulated with $1 \times 10^{-5}$ M NE, a further 10% increase could be observed (data not shown). Similar results were obtained in 5H cells (Fig. 7, *arrow 2*).

*Effects of Adrenergic Receptor Inhibitors on NE-stimulated $Ca^{2+}$ Levels and Iodide Fluxes*—The effects of both α- and β-adrenergic blocking agents on the NE- and TSH-dependent increase in $Ca^{2+}$ are summarized in Fig. 6. In these experiments, the inhibitors were added 30 s prior to NE or TSH.

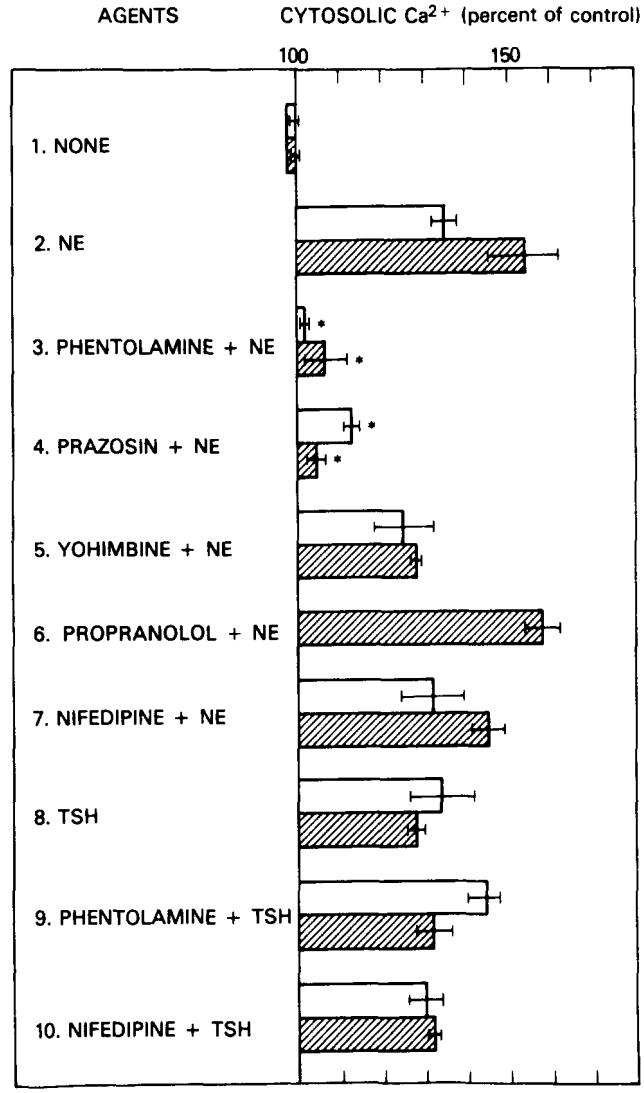


FIG. 6. **Effects of NE adrenergic blockers, TSH, and nifedipine on the cytosolic $Ca^{2+}$ level in FRTL-5 cells.** The $Ca^{2+}$ concentration was derived from the fluorescence signal of Quin2 loaded cells, as detailed under "Experimental Procedures." All agents were added at the final concentrations and in the order indicated in the agent column. The data are expressed as per cent of the control ($Ca^{2+}$ level before any addition). Both 5H (*open bars*) and 6H (*shaded bars*) FRTL-5 cells were analyzed. The data are presented as the mean of 3–10 determinations ± S.E. and again at least three different cell preparations were used. TSH was 0.1 μM in all cases. NE was at a 10 μM concentration except in the experiments with yohimbine and prazosin where NE was 1 μM. The inhibitor concentrations were as follows: phentolamine, 10 μM; prazosin, 10 nM; yohimbine, 1 μM; propranolol, 10 μM. The nifedipine concentration was 1 μM. The * denotes significant inhibition ($p < 0.005$).

The α-blocker phentolamine at $1 \times 10^{-5}$ M concentration practically abolished the NE stimulation (Fig. 6). The $\alpha_2$ blocker yohimbine had a minimal effect on the NE-dependent $Ca^{2+}$ increase at $1 \times 10^{-7}$ M concentration, whereas the specific $\alpha_1$ blocker prazosin at $1 \times 10^{-8}$ M markedly inhibited the NE effect (Fig. 6). The β-adrenergic blocker propranolol ($1 \times 10^{-5}$ M), did not affect the NE-stimulated increase in cytosolic $Ca^{2+}$.

Similar results were obtained when the adrenergic blockers were added to the 6H or 5H cells already exposed to NE. When prazosin was added to a cell suspension previously exposed to NE (Fig. 3, *arrow 1*), the effect of the NE was partially abolished. A second addition of prazosin at a higher

concentration (*arrow 2*) further reduced the $Ca^{2+}$ level. The addition of phentolamine, an $\alpha$ blocking agent, completely abolished the NE effect (*arrow 3* in Fig. 3). Similar results using both $\alpha$- and $\beta$-adrenergic blockers were obtained in 5H cells (Fig. 4).

The TSH-mediated changes in cytosolic $Ca^{2+}$ were not affected by any of the above inhibitors (see, for example, phentolamine + TSH in Fig. 6).

The NE-stimulated iodide efflux can be measured by its ability to decrease the steady-state level of iodide (Table II) (8–10). $1 \times 10^{-5}$ M propranolol had no effect on this process whereas $1 \times 10^{-5}$ M phentolamine inhibited the NE-induced decrease (Table II). Prazosin ($1 \times 10^{-7}$ M) but not yohimbine ($1 \times 10^{-6}$ M) also inhibits this NE-induced decrease (Table II). Like cytosolic $Ca^{2+}$, the effect of NE on the iodide efflux appears to be mediated by an $\alpha_1$-adrenergic receptor.

*Possible Mechanism of Increased Cytosolic $Ca^{2+}$ by TSH or NE*—Two main pathways may account for increases in cytosolic $Ca^{2+}$. One is the opening of channels in the cell membrane, thereby increasing the $Ca^{2+}$ by translocation from outside into the cell. The other pathway involves the release of the $Ca^{2+}$ from an intracellular source.

The addition of the $Ca^{2+}$ channel blocker, nifedipine (26), did not affect the NE- and the TSH-induced increase in $Ca^{2+}$ level (Figs. 6 and 7). The NE-induced decrease in steady-state level of iodide uptake also was unaffected by nifedipine (data not shown).

TABLE II

*Effect of adrenergic blocking drugs on the steady-state iodide content of FRTL-5 cells in response to norepinephrine*

| Agents | Steady-state iodide content[a] |
|---|---|
| | *% control* |
| Control | 100% |
| NE (1 $\mu$M) | 52 ± 2[b] |
| + DL-Propranolol (10 $\mu$M) | 49 ± 6 |
| + Phentolamine (10 $\mu$M) | 88 ± 7 |
| + Prazosin (0.1 $\mu$M) | 93 ± 3 |
| + Yohimbine (1 $\mu$M) | 55 ± 3 |

[a] FRTL-5 cells were incubated at 37 °C in buffered HBSS containing 10 $\mu$M NaI (10–20 mCi/mmol). At 45 min, adrenergic blocking drugs were added at the indicated final concentrations. NE (1 $\mu$M) was added 5 min later, the incubations proceeded for an additional 10 min, and the iodide content of the cells was determined as previously described (8–10). The control value was 35 pmol/$\mu$g DNA.

[b] Mean ± S.E. of three determinations.

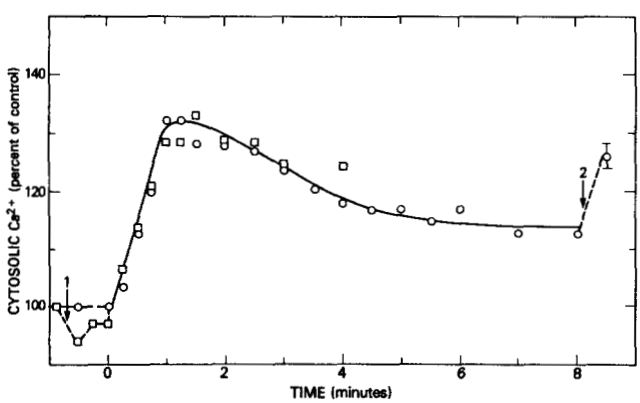


FIG. 7. **The effect of nifedipine ($1 \times 10^{-6}$ M) on the ability of NE ($1 \times 10^{-5}$ M) to increase the cytosolic $Ca^{2+}$ concentration of FRTL-5 (5H) cells in suspension** (○). Nifedipine, a $Ca^{2+}$ channel blocker, was added at *arrow 1* to one of two duplicate cell preparations; NE was added at zero time to both suspensions, and the time course of the NE effect in the presence (□) and absence (○) of nifedipine was followed. *Arrow 2* indicates the addition of $1 \times 10^{-7}$ M TSH.

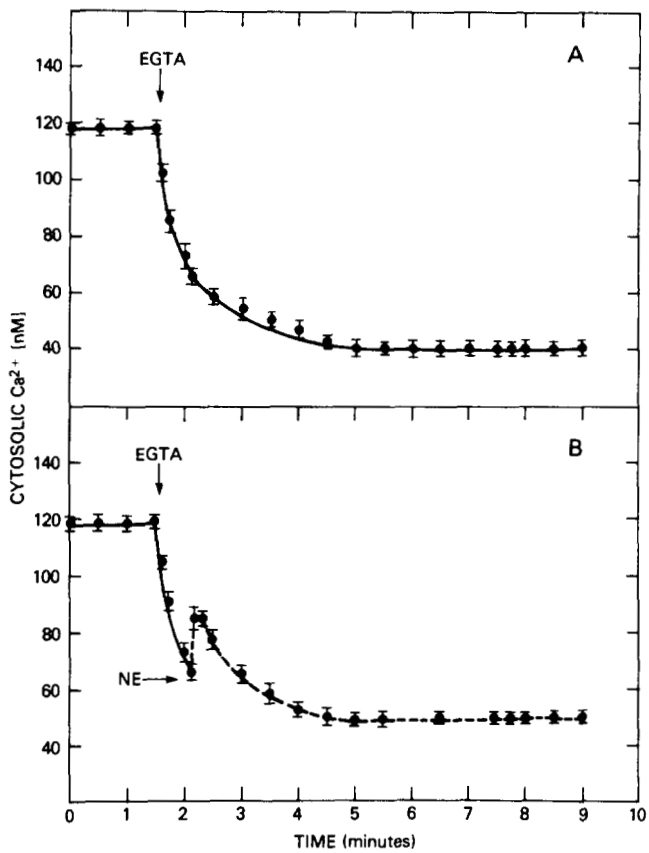


FIG. 8. **Changes in cytosolic $Ca^{2+}$ level upon addition of 6 mM EGTA in a suspension of 6H cells.** The $Ca^{2+}$ concentration in Quin2 loaded cells was calculated as detailed under "Experimental Procedures." The addition of EGTA causes the decrease of the intracellular $Ca^{2+}$ to about 40 nM (*panels A* and *B*). The addition of $1 \times 10^{-5}$ M NE immediately after EGTA treatment (~30 s, after EGTA, in *panel B*) continued to result in an increase in cytosolic $Ca^{2+}$.

When the extracellular $Ca^{2+}$ was removed by the addition of 6 mM EGTA, the cytosolic $Ca^{2+}$ levels rapidly decreased and reached a steady-state level of about 40 nM after ~4 min (Fig. 8, *panel A*). When $1 \times 10^{-5}$ M NE was added ~15–30 s after EGTA, a significant increase in the $Ca^{2+}$ level was observed (Fig. 8, *panel B*). This response was detected as long as the NE was present within 3 min from the time of EGTA addition. If NE was added in the steady-state period after EGTA (at 9 min, for example), it had no effect on intracellular $Ca^{2+}$ (data not shown). Thus, the increase in cytosolic $Ca^{2+}$ induced by NE is still apparent (Fig. 8), after the addition of EGTA even when the extracellular free $Ca^{2+}$ pool is immediately (<1 s) abolished, as evidenced in separate experiments where EGTA was added to $Ca^{2+}$ solutions containing Quin2. The loss of NE-induced activity after 3 min is presumed to reflect an EGTA action on the intracellular $Ca^{2+}$ pool (see "Discussion").

Finally, current experiments showed that neither NE nor TSH increase radiolabeled $Ca^{2+}$ entry into FRTL-5.[2] The inability of TSH to increase $Ca^{2+}$ uptake in rat thyroid hemilobes has been reported previously (27).

## DISCUSSION

The exposure of FRTL-5 cells to either TSH or NE is followed by an increase in the cytosolic $Ca^{2+}$ level as measured

[2] E. F. Grollman, manuscript in preparation.

by Quin2 fluorescence changes in this report. The effect of NE on cytosolic $Ca^{2+}$ in FRTL cells, as determined by electrophysiological methods, has been reported by Sinback and Coon (28). The increase in $Ca^{2+}$ levels by NE and TSH described in this report is dose-dependent (see Figs. 2 and 5) and is observed both in 6H and 5H cells. NE induced a rapid increase in the $Ca^{2+}$ level (maximal $Ca^{2+}$ increase in ~60 s) (see Figs. 4 and 5), whereas the maximal response to the TSH addition was obtained in 3–5 min (Fig. 1). The $Ca^{2+}$ changes induced by NE do not seem linked to a cAMP signal, since NE does not increase cAMP levels in rat FRTL-5 thyroid cells. Similarly, the TSH-induced cytosolic $Ca^{2+}$ increase also does not seem to involve a cAMP-mediated mechanism. Thus, in 5H cells changes in cytosolic $Ca^{2+}$ occur at concentrations different from those necessary to increase cAMP levels. In addition, the effect of TSH on cytosolic $Ca^{2+}$ levels is independent of the cAMP level in 6H cells. The NE-mediated increase in intracellular $Ca^{2+}$ involves $\alpha_1$- but not $\alpha_2$- or $\beta$-adrenergic receptors, whereas the TSH-stimulated increase in intracellular $Ca^{2+}$ is independent of an adrenergic receptor.

The TSH concentrations necessary to affect the cytosolic $Ca^{2+}$ level are very high relative to the "more physiological" TSH concentrations necessary for inducing increases in cAMP levels. However, pretreatment of the cells with physiological levels of TSH has a marked effect on cytosolic $Ca^{2+}$ induced by NE (Fig. 5). The change in shape of the NE curve as well as the more pronounced increase in cytosolic $Ca^{2+}$ indicate a cooperative effect resulting in a maximal response over a narrow concentration range. The NE effect, thus, behaves almost as an on-off signal over a concentration range within the physiological levels of this catecholamine.

The increase in cytosolic $Ca^{2+}$ induced by NE is at least in part derived from intracellular $Ca^{2+}$ stores. The absence of an effect by the $Ca^{2+}$ blocking agent nifedipine suggests that $Ca^{2+}$ channels are not involved in the NE action. More important, the NE-stimulated $Ca^{2+}$ increase can still be observed in the presence of EGTA, at concentrations that substantially reduce the level of free $Ca^{2+}$ in the medium. The size of the increase is 40% of that seen under normal conditions and far exceeds the increase that one might expect if it originated from any residual extracellular $Ca^{2+}$ remaining after addition of EGTA. The observation that NE cannot augment intracellular $Ca^{2+}$ when more than 3 min have elapsed after EGTA addition can be explained in either of two ways. One possibility is that the intracellular $Ca^{2+}$ stores which exchange with extracellular $Ca^{2+}$ are depleted after 3 min of exposure to EGTA. The other possibility is that a low cytosolic $Ca^{2+}$ level resultant from EGTA treatment prevents adrenergic receptor-mediated phospholipase C action. This is a key enzyme in the increase in phosphatidylinositol turnover, a process which has been proposed to be the focal point of the receptor-mediated $Ca^{2+}$ increase in several cellular systems (2).

The respective NE and TSH effects on iodide efflux and intracellular $Ca^{2+}$ are similar both with respect to time of maximal effect and ligand concentration (this report and Ref. 10). For example, the NE effect in both cases is maximal within 1 min whereas the TSH effect is maximal in 2 to 3 min. The NE effect on iodide efflux as well as cytosolic $Ca^{2+}$ involves $\alpha_1$-adrenergic receptor activation.

Since arachidonic acid can induce iodide efflux, it has been postulated (10) that NE and TSH initiate the following sequence of events to effect iodide efflux: altered phosphoinositide metabolism, an increase in cytosolic $Ca^{2+}$, $Ca^{2+}$-induced activation of phospholipase $A_2$ activity, and the breakdown of phospholipids to arachidonic acid which, either by itself or via a metabolite, is involved in the signal for iodide efflux. A preliminary report has documented NE and TSH effects on phosphoinositide metabolism, *i.e.* phosphatidylinositol 1,4-bisphosphate conversion to phosphatidylinositol 1,4,5-trisphosphate (29). The present report also finds an increase in cytosolic $Ca^{2+}$ by both ligands. Melittin, a peptide which directly activates phospholipase activity, was found to stimulate iodide efflux;[3] 5,8,11,14-eicosatetraenoic acid a cyclooxygenase and lipoxygenase inhibitor but not indomethacin, a cyclooxygenase blocker, blocks NE and TSH stimulated efflux, suggesting that a lipoxygenase metabolite of arachidonic acid is involved in the receptor-mediated increase in iodide efflux. In sum, the link of increased cytosolic $Ca^{2+}$ in FRTL-5 cells by NE and TSH to NE- and TSH-stimulated iodide efflux in FRTL-5 cells seems reasonable. The data do not, however, define the mechanism by which the linkage is obtained. Thus, the importance of coordinate changes in diacylglycerol and signal involvement via a kinase C mechanism cannot be excluded, particularly given the low level of the $Ca^{2+}$ increase relative to increases in other systems involving extracellular $Ca^{2+}$ pools.

The *in vivo* mechanism of thyroglobulin formation involves the uptake of iodide through the basal membrane, its transport across the cell, and its release in the lumen, where the iodination of thyroglobulin predominantly occurs (6, 7). The first process is TSH-stimulated, cAMP-dependent, and cannot be mimicked by NE. The second process is also TSH-stimulated, but is mimicked by NE or A23187 and it is non-cAMP-dependent (30).

In FRTL-5 thyroid cells, NE significantly increases iodide efflux (10) and iodination of thyroglobulin.[4] Without NE, the thyroglobulin secreted by these cells into the media is poorly iodinated. Since FRTL-5 cells exposed to high concentrations of exogeneously added iodide do not iodinate thyroglobulin, the iodide efflux induced by NE/TSH may represent a specific pool of iodide necessary for the iodination of thyroglobulin. NE-induced iodide efflux and iodination of thyroglobulin in FRTL-5 cells therefore have properties similar to the process where iodide is transported into the follicle lumen *in vivo* (30). Under these circumstances, it is reasonable to suggest that iodide release in the lumen may be regulated by phosphoinositide metabolism, is triggered by an increase in the $Ca^{2+}$ level in the thyroid cell, and may, under physiological conditions, represent a concerted, co-regulated action of NE and TSH.

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