Phosphatase Inhibition Reveals a Calcium Entry Pathway Dependent on Protein Kinase A in Thyroid FRTL-5 Cells

COMPARISON WITH STORE-OPERATED CALCIUM ENTRY*

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Calcium entry through store-operated calcium channels is an important entry mechanism. In the present report we have described a novel calcium entry pathway that is independent of depletion of intracellular calcium stores. Treatment of the cells with the phosphatase inhibitor calyculin A (caly A), which blocked thapsigargin-evoked store-operated calcium entry (SOCE), induced a potent concentration-dependent calcium entry. In a calcium-free buffer, acute addition of caly A evoked a very modest increase in cytosolic free calcium (Ca²⁺). This increase was not from the agonist-mobilizable calcium stores, as the thapsigargin-evoked increase in [Ca²⁺], was unaltered in caly A-treated cells. The caly A-evoked calcium entry was not blocked by Gd³⁺ or 2-APB, whereas SOCE was. Caly A enhanced the entry of barium, indicating that the increase in intracellular calcium was not the result of a decreased extrusion of calcium from the cytosol. Jaspilokinolide and cytochalasin D had only marginal effects on calcium entry. The protein kinase A (PKA) inhibitor H-89 and an inhibitory peptide for PKA abolished the caly A-evoked entry of both calcium and barium. The SOCE was, however, enhanced in cells treated with H-89. In cells grown in the absence of thyrotropin (TSH), the caly A-evoked entry of calcium was smaller compared with cells grown in TSH-containing buffer. Stimulation of cells grown without TSH with forskolin or TSH restored the calyculin A-evoked calcium entry to that seen in cells grown in TSH-containing buffer. SOCE was decreased in these cells. Our results thus suggest that TSH, through the production of cAMP and activation of PKA, regulates a calcium entry pathway in thyroid cells. The pathway is distinctly different from the SOCE. As TSH is the main regulator of thyroid cells, we suggest that the novel calcium entry pathway participates in the regulation of basal calcium levels in thyroid cells.

Calcium is the key regulator of a multitude of cellular processes, including proliferation, gene transcription, and apoptosis (1). To enable such a broad spectrum of different actions, the cells have evolved a plethora of different mechanisms regulating cellular calcium levels. Calcium may be mobilized from intracellular compartments by the activation of inositol 1,4,5-trisphosphate (IP₃) receptors or ryanodine receptors. Furthermore, calcium may enter the cells through a multitude of calcium channels, both voltage-dependent and -independent channels. Ca²⁺ ATPases in both the plasma membrane and in intracellular membranes participate in regulation by transporting calcium out of the cell or into intracellular compartments. In non-excitable cells, calcium entry is usually evoked as a result of agonist-mediated activation of the phospholipase C/IP₃ pathway, mobilization of sequestered calcium from intracellular stores, and, as a consequence of this depletion, store-operated calcium entry (SOCE). Calcium entry can also be the result of agonist-evoked activation of receptor channels (i.e. the P2X receptors for ATP) or the activation of non-selective cation channels (i.e. second-messenger channels and stretch-activated channels). Of these different calcium entry pathways, the activation of store-operated calcium channels has attracted substantial interest (2, 3). In addition, several investigations show that agonist-evoked calcium entry (e.g. as seen in calcium oscillations) is mediated through a distinct, arachidonic acid-regulated calcium channel (4, 5).

The SOCE, also called the capacitative calcium entry, is the direct result of depletion of intracellular calcium stores. The emptying of the stores evoked a profound entry of calcium into the cytosol from the extracellular space, through mechanisms not yet fully understood. Three theories are presently at hand. First, the emptying of intracellular stores produced a factor that diffuses to the plasma membrane and opens calcium channels. Second, emptying of the calcium stores causes the fusion of vesicles containing calcium channels with the plasma membrane. In the last model, the depletion of calcium stores causes a conformational coupling of the IP₃ receptor with calcium channels (2, 3). Very convincing evidence has been obtained in support of the conformational coupling theory, e.g. the disruption of the actin cytoskeleton potently abrogates store-operated calcium entry (6, 7). Thus, the disruption and rearrangement of the cytoskeleton would physically hinder a coupling between the IP₃ receptor and calcium channels located on the plasma membrane. However, evidence obtained in several investigations has provided support for all three mechanisms. The situation is not made easier to fathom considering the wealth of different types of calcium channels (in particular the TRP

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‡ The abbreviations used are: IP₃, inositol 1,4,5-trisphosphate; PKA, protein kinase A; PKI₆₈₋ₓ₂₀, inhibitory peptide for PKA; PBS, phosphate-buffered saline; [Ca²⁺], cytosolic calcium concentration; SOCE, store-operated calcium entry; TRP channels, transient receptor potential channels; TSH, thyrotropin;
family of channels) (8) that have been suggested to mediate, at least in part, store-operated calcium entry.

In thyroid FRTL-5 cells, changes in intracellular calcium control several processes, including the regulation of iodide efflux (9–11) and the regulation of proliferation and synthesis of DNA (12, 13). Furthermore, changes in intracellular calcium may modify the TSH-evoked effects in thyroid cells (9, 14). In addition to agonist-evoked changes in Ca\(^{2+}\) (15), the SOCE pathway is probably an important regulator of intracellular calcium signaling (16). In preliminary experiments aimed at further understanding the mechanisms regulating calcium entry, we observed that the phosphatase inhibitor calyculin A, a potent disrupter of the actin cytoskeleton, abolished SOCE in our cells. However, in those experiments we observed that the resting calcium levels were higher compared with that seen in control cells. Further investigations revealed that calyculin A evoked a substantial calcium entry that was distinct from the SOCE. Furthermore, the calyculin A-evoked calcium entry appeared to be crucially dependent on cAMP and PKA. As the cAMP-PKA pathway is a major regulator of thyroid cell function, we suggest that the calcium entry mechanism we have described in the present investigation is of importance in regulating calcium levels in thyroid cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Culture medium, serum, and hormones were purchased from Invitrogen. Biological Industries (Beth Haemek, Israel), and Sigma. Culture dishes were obtained from Falcon Plastics (Oxnard, CA). Calyculin A, H-89, forskolin, fluorescein isothiocyanate-labeled phalloidin, jasplakinolide, isobutylmethylxanthin, and cytochalasin D were purchased from Sigma. Calphostin C was from Alexis Corporation (Laufelfingen, Switzerland). The myristoylated inhibitory peptide for PKA, PKI14–22, was from Biomol (Plymouth Meeting, PA). GF109203X (Laufelfingen, Switzerland) was purchased from Molecular Probes, Inc. (Eugene, OR). Thapsigargin was from LC Services Corp. (Woburn, MA). [\(^3\)H]CAMP (25 Ci/mmol) was from PerkinElmer Life Sciences. All other chemicals used were of reagent grade. Bovine TSH was a generous gift from the National Hormone and Pituitary Program (National Institutes of Health, Bethesda, MD).

**Cell Culture**—Rat thyroid FRTL-5 cells, originally obtained from the Interthyr Foundation (Bethesda, MD), were grown in Coon's modified Ham's F-12 medium supplemented with 5% calf serum and six hormones (6H) (17) (insulin, 10 μg/ml; transferrin, 5 μg/ml; hydrocortisone, 10 nM; tripeptide Gly L-His L-Lys, 10 nM; TSH, 0.3 milliunits/ml; somatomedin, 10 nM) in a water-saturated atmosphere of 5% CO\(_2\) and 95% air at 37 °C. Prior to an experiment, cells from one donor culture dish were harvested using 0.02% EDTA-0.1% trypsin solution and subcultured on coverslips on 100-mm culture dishes (Falcon Plastics). The cells were grown for 48 h at a density of 10^6 cells/tube (18) using the patch clamp whole-cell technique in voltage and phosphodiesterase activity. Then aliquots of cells (0.5 × 10^6 cells/tube) were stimulated with 140 nM calyculin A for 15 and 30 min. Control cells were treated with vehicle only. The coverslips were then washed twice with warm (37 °C) PBS. The fixation of the cells was made by incubating the coverslips for 10 min in 3.7% formaldehyde at room temperature, after which the cells were washed twice with PBS. The cells were permeabilized for 3–5 min in 0.1% Triton X-100 in PBS and washed twice with warm PBS. Prior to fluorescence staining, the cells were incubated with PBS containing 1% bovine serum albumin for 30 min at room temperature. The fluorescein isothiocyanate-conjugated phalloidin (300 units; Molecular Probes) was diluted 1:40 with PBS-bovine serum albumin, and the cells were incubated with the solution for 20 min at room temperature. The cells were then washed twice with warm PBS.

The cells were examined using a Leica TCS SP confocal microscope equipped with a 488-nm argon laser (Olympus, Melville, NY) and a 40× oil objective. All figures were acquired with Leica TCS NT software.

**Measurement of Cellular Ca\(^{2+}\) Levels**—The cells were harvested as described for the calcium experiments and were preincubated in HBSS for 20 min in the presence of 0.3 mM isobutylmethylxanthin (to inhibit phosphodiesterase activity). Then aliquots of cells (0.5 × 10^6 cells/tube) were stimulated with 100 nM calyculin A. After 15 min the reaction was stopped with perchloric acid to a final concentration of 0.5 M. The samples were neutralized with KOH, and the Ca\(^{2+}\) concentrations were determined using a protein binding method (19).

**Confocal Microscopy of Actin Filaments**—In the studies the coverslips were performed using the patch clamp whole-cell technique in voltage and current clamp mode (20). Prior to the experiments, the cells were incubated with 0.02% EDTA-trypsin solution and subcultured on coverslips on 24-well plates (BD Pharmingen) for 2–5 days. The coverslips were placed in a perfusion chamber. During recordings the cells were continuously perfused with a standard solution containing (in mM): NaCl, 150; KCl, 5; MgCl\(_2\), 1; CaCl\(_2\), 1.8; HEPES, 5 (pH adjusted to 7.4 with KOH). All recordings were made at room temperature. Currents were recorded with an EPC-9 amplifier (HEKA, Lambrecht, Germany). Analyses of the recordings were made using Pulse and Pulse Fit software (HEKA).

**Statistics**—The results are expressed as the mean ± S.E. Statistical analysis was made using Student’s t test for paired observations. When three or more means were tested, analysis of variance was used.

**RESULTS**

**Calcium Entry Mechanisms in FRTL-5 Cells**—Previous studies have shown that FRTL-5 cells respond to depletion of intracellular calcium stores with a substantial entry of calcium (16). In the present study we have shown that the thapsigargin-evoked depletion of calcium stores results in entry of exog-
nous calcium and that this entry can be blocked by 30 μM 2-APB, 1 μM Gd3+ and by pretreatment of the cells with 100 nM of the phosphatase inhibitor calyculin A (Fig. 1, A and B).

We observed that the basal calcium level of cells treated with calyculin A always was higher than in control cells (218 ± 5 nm and 126 ± 5 in calyculin-treated and control cells, respectively, n = 6, p < 0.05, see Fig. 1A). To investigate the reason for this, we stimulated FRTL-5 cells with calyculin A and observed a distinct increase in [Ca2+]i (Fig. 2A). This increase was not blocked by either 30 μM 2-APB or 1 μM Gd3+ (Fig. 2B). The results thus suggest that, in FRTL-5 cells, calyculin A evokes an increase in [Ca2+]i, that does not occur through store-operated calcium channels. Calyculin A per se evoked a very modest increase in [Ca2+]i, that is dependent on the concentration of calyculin A (Fig. 2C), in cells in a calcium-free buffer (22 ± 5 nm, n = 4). However, calyculin A did not mobilize calcium from IP3-dependent stores, as the thapsigargin-evoked release of sequestered calcium in a calcium-free buffer was 151 ± 27 nm in calyculin A-treated cells and 148 ± 9 nm in control cells, respectively.

To further investigate the effect of calyculin A, we pretreated the cells with calyculin A and suspended the cells in a calcium-free buffer. Then calcium (final concentration 1 mM) was added back to the cells. In these experiments, we observed an increase in [Ca2+]i that was dependent on the concentration of calyculin A (Fig. 2, C and D). The increase in [Ca2+]i, was insensitive to both 2-APB and Gd3+ (Fig. 2E). In contrast to this observation, if FRTL-5 cells were stimulated with thapsigargin and calcium was then readded, the calcium entry was potently abrogated by 2-APB and Gd3+ (Fig. 3A). However, if cells treated with 100 nm calyculin A were stimulated with 1 μM thapsigargin in a calcium-free buffer and calcium was readded, we always obtained a substantial entry of calcium. The initial calcium entry was indistinguishable from that seen in cells stimulated with thapsigargin only (867 ± 91 nm and 817 ± 69 nm in thapsigargin- and calyculin A-treated cells, respectively; Fig. 3B). The increase in [Ca2+]i then slowly declined to a plateau level lower than that seen in cells stimulated with thapsigargin only. The new plateau level of [Ca2+]i was 495 ± 35 nm in control cells and 349 ± 32 nm in calyculin A-treated cells (p < 0.05, Fig. 3B). The increase in [Ca2+]i, probably is a combination of the calyculin-evoked calcium entry and a small residual store-operated calcium entry due to the thapsigargin-evoked depletion of the endoplasmic reticulum calcium stores. A comparison of the increase in [Ca2+]i, obtained after addition of calcium to cells treated with calyculin only, or with both calyculin and thapsigargin, is shown in Fig. 3D.

To investigate the effect of calyculin A on the actin cytoskeleton in our cells, we labeled control cells and cells treated with 100 nM calyculin with fluorescein isothiocyanate-conjugated phalloidin. As can be seen in Fig. 4, treatment of the cells resulted in a dramatic reconstruction of the actin filaments in the cells. The effect of calyculin A in our cells was very similar to that observed in other cell types (see Refs. 6 and 7) and thus probably explains why the thapsigargin-evoked SOCE was attenuated in our cells after treatment with calyculin A.

To ensure that calyculin A actually enhanced calcium entry and not increased [Ca2+]i, by blocking plasma membrane Ca2+ ATPases and calcium extrusion, we measured the entry of Ba2+ in calyculin A-stimulated cells. In these experiments, pretreatment with calyculin A clearly enhanced Ba2+ entry (Fig. 5A). For comparison, the thapsigargin-evoked Ba2+ entry is shown (Fig. 5B). The results in Fig. 5 show that the calyculin A-evoked Ba2+ entry (49 ± 3 fluorescent units/30 s) is more pronounced than the thapsigargin-evoked Ba2+ entry (31 ± 4 fluorescent units/30 s; p < 0.05). Furthermore, both calyculin A and thapsigargin enhanced the entry of Sr2+ (results not shown).

Mechanisms of Calyculin A-evoked Calcium Entry—Calyculin A could theoretically enhance calcium entry by hyperpolarizing the membrane potential, thus increasing the electrochemical driving force for calcium. However, in patch clamp experiments in the current clamp mode, we could not show an effect of calyculin A on the membrane potential compared with vehicle-treated cells. The change in E膜 in control cells was −5.6 ± 3.6 mV, compared with −7.7 ± 0.8 mV in cells treated with 100 nM calyculin A as measured over a time span of 6 min.

Calyculin A potently redistributes the actin filaments of the cytoskeleton. To test whether the effects of calyculin A were the result of this redistribution, we treated our cells with either
jasplakinolide (3 μM for 60 min) or cytochalasin D (5 μM for 30 min). Addition of calcium to cells treated with jasplakinolide or cytochalasin D evoked a transient increase in \([Ca^{2+}]_i\) (248 ± 66 and 225 ± 12 nM, respectively) that was not significantly different from that seen in control cells (184 ± 17 nM).

We also tested whether cyclosporin, an inhibitor of the phosphatase calcineurin, could mimic the effect of calyculin A. However, incubating the cells with cyclosporin (1 μg/ml for 15 min) did not enhance calcium entry (data not shown).

We have previously shown that protein kinase C can potently modulate calcium entry in FRTL-5 cells (16). Pretreating the cells with either calphostin C (100 nM) or GF109203X (10 μM) for 15 min did not attenuate the calyculin A-evoked calcium entry (data not shown). In addition, stimulating calyculin A-pretreated cells with 100 nM phorbol 12-myristate 13-acetate did not increase the calcium entry compared with control cells treated with calyculin A only (data not shown). However, when the cells were pretreated with the PKA inhibitor H-89 (10 μM for 15 min) or with PKI14–22 (10 μM for 30 min), both the calyculin A-evoked calcium and barium entry were abolished (Fig. 6, A–C). In sharp contrast to this, we observed that the thapsigargin-evoked calcium entry was enhanced in the presence of 10 μM H-89 (Fig. 6D).

To further test the hypothesis that PKA was involved in regulating the calyculin A-evoked calcium entry, we cultured our cells in medium lacking TSH. The calyculin A-evoked calcium entry was significantly reduced in the cells (334 ± 31 nM) compared with cells grown in the presence of TSH (571 ± 47 nM, p < 0.05). If TSH-depleted cells were stimulated with a low dose of forskolin (0.1 μM), the calyculin A-evoked calcium entry was enhanced significantly (507 ± 39 nM, p < 0.05) compared with TSH-depleted control cells. Furthermore, if the cells were stimulated with 0.3 milliunits of TSH, the calyculin A-evoked increase in \([Ca^{2+}]_i\) (702 ± 60 nM) was significantly (p < 0.05)

**FIG. 2. Calyculin A-evoked calcium entry.** A, cells were stimulated with 100 nM calyculin A, and the change in \([Ca^{2+}]_i\) was measured. B, the calyculin A-evoked increase in \([Ca^{2+}]_i\) was not blocked by either 30 μM 2-APB or 1 μM Gd^{3+}. Each bar is the mean ± S.E. of four to separate experiments. C, cells pretreated with 100 nM calyculin A for 15 min (trace a) were added to a calcium-free buffer, and then calcium (final concentration 1 mM) was added. Trace b shows addition of calcium to control cells not treated with calyculin A. D, concentration-dependent effect of calyculin A on calcium entry. Cells pretreated for 15 min with the indicated concentrations of calyculin A were resuspended in a calcium-free buffer, and then 1 mM calcium was added. Each data point gives the mean ± S.E. of four experiments. E, lack of an effect of 30 μM 2-APB or 1 μM Gd^{3+} on the increase in \([Ca^{2+}]_i\), obtained after adding 1 mM calcium to calyculin A-treated cells (100 nM for 15 min) in a calcium-free buffer. Each bar gives the mean ± S.E. of four separate experiments.
increased compared with TSH-depleted control cells (Fig. 7, B and C). Thus, both forskolin and TSH restored the calyculin A-evoked calcium entry to the same level as seen in cells grown in the presence of 0.3 milliunits of TSH.

To investigate whether a direct activation of PKA without prior treatment with calyculin A could enhance calcium entry in TSH-depleted cells, we stimulated cells with 10 μM forskolin and calcium. As can be seen in Fig. 7D, forskolin evoked an entry of calcium (150 ± 7 nM) that was significantly smaller in cells pretreated with 10 μM H-89 (109 ± 2 nM, p <0.05).

We next investigated whether calyculin A increased cellular cAMP levels in FRTL-5 cells. The concentration of cAMP in cells stimulated with 100 nM calyculin A for 15 min was 47 ± 0.5 pg/0.5 × 10⁶ cells (n = 4), compared with 6.2 ± 1.0 pg/0.5 × 10⁶ (n = 4, p >0.05) in vehicle-treated control cells. Thus, the effect of calyculin A was downstream from the production of cAMP in the cells, suggesting that the effect of calyculin A was the result of an enhanced phosphorylation by PKA.

For comparison, the thapsigargin-evoked increase in [Ca²⁺], was also measured in TSH-depleted cells. Our results show that the thapsigargin-evoked SOCE was enhanced in cells grown in the absence of TSH compared with cells stimulated with 0.3 mM TSH (Fig. 7, E and F). Thus, an increase in cAMP has an antagonizing effect on SOCE in FRTL-5 cells.

We also wanted to investigate whether calcium entry evoked by G protein-coupled receptors could be enhanced by treatment with calyculin A. When cells were pretreated with 100 nM calyculin A for 15 min, the calcium signal evoked by 10 μM ATP was clearly broadened (Fig. 8). The decrease in [Ca²⁺], was 36 ± 5 nM/10 s at 75% of the downward part of the calcium
Phosphatase Inhibition Enhances Ca^{2+} Entry

In the present investigation we have shown that, in addition to store-operated calcium entry (16), thyroid FRTL-5 cells also have a previously unknown calcium entry pathway. This calcium entry pathway seems to depend on the activation of PKA. The novel entry pathway is distinctly different from the SOCE pathway. We have based our conclusion on the following observations. Store-operated calcium entry was almost totally blocked by preincubating the cells with calyculin A, whereas the novel calcium entry pathway was revealed by inhibition of phosphatases with calyculin A. The increase in [Ca^{2+}]i evoked by calyculin A was the result of calcium entry, not a decreased extrusion of calcium, as calyculin A potently enhanced the calcium entry pathway seems to depend on the activation of PKA.

Effect of Calyculin A on [Ca^{2+}]i in Single Cells—As the above experiments were performed with cells in suspension, we wanted to investigate whether calyculin A also enhanced calcium entry in single cells. As can be seen in Fig. 9, pretreatment of single cells with 100 nM calyculin A for 15 min enhanced calcium entry compared with the entry obtained in control cells. The effect of calyculin A was not altered by the presence of 1 μM Gd^{3+}, whereas the thapsigargin-evoked calcium entry was decreased (data not shown) in a manner similar to that observed in cells in suspension.

**FIG. 5.** Comparison of the calyculin A- and thapsigargin-evoked increases in [Ba^{2+}]i. A, cells treated with 100 nM calyculin A for 15 min were resuspended in a calcium-free buffer, and then 1 mM Ba^{2+} was added (trace a). Control cells, trace b. B, cells in a calcium-free buffer were stimulated with 1 μM thapsigargin (Tg), and then 1 mM Ba^{2+} was added (trace a). Control cells, trace b. Each experiment was repeated at least four times.

**FIG. 6.** Effects of H-89 and PKI14–22 on calyculin A- and thapsigargin-evoked calcium entry. A, calyculin A-treated cells (100 nM for 15 min; trace a) or control cells (trace b) were suspended in a calcium-free buffer containing 10 μM H-89, or the cells were treated with calyculin A and PKI14–22 (10 μM for 30 min; trace c). Calcium (final concentration 1 mM) was then added. B, cells treated with calyculin A (100 nM for 15 min) were resuspended in a calcium-free buffer containing vehicle (Control) or 10 μM H-89 (H-89) or were pretreated with calyculin and PKI14–22 (10 μM for 30 min). Calcium (final concentration 1 mM) was then added. Each bar gives the mean ± S.E. of three to six separate experiments; *, p < 0.05. C, calyculin A-treated cells (100 nM for 15 min; trace a) or control cells (trace b) were suspended in a calcium-free buffer containing 10 μM H-89, or the cells were treated with calyculin A and PKI14–22 (10 μM for 30 min; trace c). Barium (final concentration 1 mM) was then added. D, control cells or cells resuspended in a calcium-free buffer containing 10 μM H-89 were stimulated with thapsigargin, and then calcium (final concentration 1 mM) was added. The calcium-evoked peak and plateau in [Ca^{2+}]i were measured. Each bar gives the mean ± S.E. of four to six separate experiments; *, p < 0.05.
been explained by conformational coupling of the IP₃ receptor with plasma membrane calcium channels. Evidence suggests that these channels belong to the TRP family of channel proteins (possibly the TRPC3, Refs. 22, 23). Compelling evidence for conformational coupling has been obtained in experiments where calyculin A-evoked formation of cortical actin layers inhibited calcium entry (6, 24, 25). However, this effect was considered mainly a function of the reorganization of the actin cytoskeleton, as jasplakinolide also inhibited store-operated calcium entry. Experiments performed with cytochalasin D, another agent forming cortical actin, resulted in similar results. The present calcium entry pathway, on the other hand, is not attenuated after calyculin A-evoked cortical actin formation. Thus, our results strongly point at an effect mediated by an inhibition of a phosphatase, as treatment of the cells with jasplakinolide or cytochalasin D did not evoke a response similar to that obtained by calyculin A. Furthermore, the calcium entry was not the result of store depletion, as calyculin A did not modify agonist-evoked store depletion. Thus, the present calcium entry is probably not the result of a coupling of intracellular stores to calcium channels in the plasma membrane. We suggest that the inhibition of a phosphatase uncouples a block of a calcium entry pathway.

Previous studies have shown that a phosphatase is involved in regulating SOCE. The very first studies on SOCE showed that emptying of intracellular stores evoked a highly calcium-selective current (calcium release-activated current, I_{CRAC}) (26, 27). The activation of this current involved a phosphatase (27). In another study it was shown that the effect of a presently unknown calcium influx factor was enhanced by inhibition of

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**FIG. 7. Effect of calyculin A and thapsigargin on [Ca²⁺], in cells grown without TSH**. A, addition of calcium (final concentration 1 mM) and 0.3 mM TSH simultaneously to cells grown in TSH-free medium for 5 days (trace a). Addition of calcium only (trace b) or calcium only (trace c) was added to the cells. C, summary of several experiments performed as in panel B. In Control, calyculin A-treated cells were stimulated with calcium only, whereas in TSH the cells were stimulated with both calcium and TSH. In TSH + H-89 and TSH + PKI, the cells were pretreated with calyculin A and 10 μM H-89 or calyculin and PKI₁₄–₂₂ (10 μM for 30 min) and then stimulated with both calcium and TSH. Each bar gives the mean ± S.E. of three to five separate experiments; *, p < 0.05. D, cells grown in the absence of TSH were stimulated with thapsigargin (Tg, final concentration 1 μM), and then calcium only (trace a) or calcium and TSH (trace b) was added to the cells. The traces shown are representative of five separate experiments. E, summary of several experiments performed as in panel D. In Control, calcium only was added to cells stimulated with thapsigargin, whereas in TSH both calcium and TSH were added to cells stimulated with thapsigargin. The calcium-evoked peak and plateau levels of [Ca²⁺] were measured. Each bar gives the mean ± S.E. of five separate experiments; *, p < 0.05.
that seen in the present investigation. In addition, arachidonic acid depleted intracellular calcium stores (38). Presently no information exists regarding the sensitivity of the arachidonic acid-evoked calcium entry to calcineurin A. However, the arachidonic acid-evoked current was inhibited by calyculin A (39), whereas we were unable to evoke any calcium entry by treating the cells with cytosporin A. Trebak et al. (40) have shown that low concentrations of Gd³⁺ did not block receptor-activated HTRPC and 2-APB had only a weak effect. Thus, we cannot exclude that treatment of our cells with calyculin A reveals calcium entry through a calcium channel of the TRP family of channels. Furthermore, we cannot exclude the possibility that some (e.g. autocrine) form of receptor-mediated activation of calcium entry may occur after treatment with calcineurin A. This is unlikely, as previous studies in FRTL-5 cells have shown that calyculin A blocked the coupling of G protein-coupled receptors to at least phospholipase C (21). We did, however, observe that activation of G protein-coupled receptors evoked calcium signals that, in part, were mediated by the novel entry mechanism. These calcium signals could further be enhanced by calyculin A and were blocked by both H-89 and PKI14–22. Our results thus point at a rather complex calcium signaling pattern in response to agonists activating G protein-coupled receptors as ATP also evokes SOCE in FRTL-5 cells (15). A very interesting observation was that the calcium entry appeared to be dependent on the cAMP-PKA pathway: if PKA were blocked by H-89 or PKI14–22, the calyculin A-evoked calcium entry was blocked. Furthermore, in cells grown in the absence of TSH, the calcineurin A-evoked calcium entry was of lower magnitude compared with cells grown in the presence of TSH. Addition of TSH or forskolin to cells grown in TSH-free medium rapidly enhanced calcium entry in cells treated with calyculin, suggesting that phosphorylation of a channel protein (or an accessory protein) by PKA is involved in regulating calcium entry. In sharp contrast to this observation, we showed that the thapsigargin-evoked calcium entry was attenuated by cAMP in cells grown in TSH-free medium. A similar cAMP-evoked inhibition of store-operated calcium entry has been shown in platelets (7). The TSH-cAMP-PKA pathway is one of the most important regulators of thyroid cell function. It is tempting to suggest that the calcium entry mechanism revealed in the present investigation participates in the regulation of basal calcium levels in thyroid cells. This regulation probably is under strict control of PKA and a presently unknown phosphatase. Furthermore, the entry pathway is clearly distinct from store-operated calcium entry. Apparently G protein-coupled receptors also may utilize this calcium entry pathway. Further investigations will reveal whether a complex interplay exists between store-operated calcium entry and the novel cAMP-dependent calcium entry.

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REFERENCES

1. Berridge, M. J., Bootman, M. D., and Roderick, H. L. (2003) Nat. Rev. Mol. Cell. Biol. 4, 517–529
2. Putney, J. W., Jr., Broad, L. M., Braut, F.-J., Liewemont, J.-P., and St. J. Bird, G. (2001) J. Cell Sci. 114, 2223–2229
3. Venkatachalam, K., van Rossum, D. B., Patterson, R. L., Ma, H.-T., and Gill, D. L. (2002) Nat. Rev. Cell. Biol. 4, E263–E272
4. Shuttleworth, T. J. (1996) J. Biol. Chem. 271, 21720–21725
5. Shuttleworth, T. J., and Mignen, O. (2003) Biochem. Soc. Trans. 31, 916–919
6. Patterson, R. L., van Rossum, D. B., and Gill, D. L. (1999) Cell 98, 487–499
7. Rosado, J. A., Porras, T., Conde, M., and Sage, S. O. (2001) J. Biol. Chem. 276, 15666–15675
8. Clapham, D. E. (2003) Nature 426, 517–524
9. Weiss, S. J., Philip, N. P., and Grollman, E. F. (1984) Endocrinology 114, 1108–1113
10. Cordia, D., Marodi, R., Kohn, L. D., Axeldroj, L., and Laxin, A. (1985) J. Biol. Chem. 260, 9230–9236
11. Berman, M. I., Thomas, C. G., Jr., and Nayfeh, S. N. (1987) Mol. Cell. Endocrinol. 54, 151–163
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12. Törnquist, K., Ekokoski, E., and Dugué, B. (1996) *J. Cell. Physiol.* **166**, 241–248
13. Ekokoski, E., Webb, T. E., Simon, J., and Törnquist, K. (2001) *J. Cell. Physiol.* **187**, 166–175
14. Okajima, F., Sho, K., and Kondo, Y. (1988) *Endocrinology* **123**, 1035–1043
15. Törnquist, K. (1992) *J. Cell. Physiol.* **150**, 90–98
16. Törnquist, K. (1993) *Biochem. J.* **290**, 443–447
17. Ambesi-Impimbiugo, F. S., Parke, L. A. M., and Coon, H. G. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 3455–3459
18. Grynkiewicz, G., Poenia, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3449–3450
19. Norstedt, C., and Fredholm, B. B. (1990) *Anal. Biochem.* **189**, 231–234
20. Hamill, O. P., Marty, A., Neher, E., Sakman, B., and Sigworth, F. J. (1981) *Pfluegers Arch.* **391**, 85–100
21. Laglia, G., Zeiger, M. A., Leipricht, A., Caturegli, P., Levine, M. A., Kohn, L. D., and Saji, M. (1996) *Endocrinology* **137**, 3170–3176
22. Ma, H.-T., Patterson, R. L., van Rossum, D. B., Birnbaumer, L., Mikoshiba, K., and Gill, D. L. (2000) *Science* **287**, 1647–1651
23. Kiseliov, K. I., Xu, X., Mozhayeva, G. N., Kuo, T., Pessah, I., Mignery, G. A., Birnbaumer, L., and Muallim, S. (1998) *Nature* **396**, 478–482
24. Montero, M., Garcia-Sancho, J., and Alvarez, J. (1994) *J. Biol. Chem.* **269**, 3963–3967
25. Bosade, J. A., Jenner, S., and Sage, S. O. (2000) *J. Biol. Chem.* **275**
26. Hoth, M., and Penner, R. (1992) *Nature* **355**, 333–336
27. Parekh, A. B., Terlau, H., and Stühne, W. (1993) *Nature* **364**, 814–818
28. Randriamampita, C., and Tsien, R. Y. (1995) *J. Biol. Chem.* **270**, 29–32
29. Ma, R., Pluznick, J., Kudlacek, P., and Sansom, S. C. (2001) *J. Biol. Chem.* **276**, 25759–25765
30. Albert, A. P., and Large, W. A. (2002) *J. Physiol.* **544**, 113–125
31. Hofmann, T., Obukhov, A. G., Schaefer, M., Harteneck, C., Gudermann, T., and Schultz, G. (1999) *Nature* **397**, 259–263
32. Schuhmann, C., Romain, C., Baumgartner, W., and Groschner, K. (1997) *J. Gen. Physiol.* **110**, 503–513
33. Larsson, O., Barker, C. J., Sjöholm, A., Cataprist, H., Michell, R. H., Bertorello, A., Nilsson, T., Honkanen, R. E., Mayr, G. W., Zwillier, J., and Berggren, P. O. (1997) *Science* **278**, 471–475
34. Birnbaumer, L., Zhu, X., Jiang, M., Boulay, G., Peyton, M., Vannier, B., Brown, D., Platano, D., Stefani, E., and Birnbaumer, M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 15185–15202
35. Lalevée, N., Resin, V., Arnaudeau, S., Demaurex, N., and Rossier, M. (2003) *Endocrinology* **144**, 4575–4585
36. Shuttleworth, T. J., and Thompson, J. L. (1998) *J. Biol. Chem.* **273**, 32633–32643
37. Mignery, O., Thompson, J. L., and Shuttleworth, T. J. (2003) *J. Biol. Chem.* **278**, 10174–10181
38. Törnquist, K., Ekokoski, E., Forss, L., and Matsson, M. (1994) *Cell Calcium* **15**, 153–161
39. Mignery, O., Thompson, J. L., and Shuttleworth, T. J. (2003) *J. Biol. Chem.* **278**, 40088–40096
40. Trebak, M., St. J. Bird, G., McKay, R. R., and Putney, J. W., Jr. (2002) *J. Biol. Chem.* **277**, 21617–21623