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Na^+-ionophore, monensin-induced rise in cytoplasmic free calcium depends on the presence of extracellular calcium in FRTL-5 rat thyroid cells *

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Calcium is an important regulator of cell function, and may be influenced by the intracellular sodium content. In the present study, the Na^+-ionophore, monensin, was used to investigate the interrelationship between changes in intracellular Na^+ concentration ($[Na^+]_i$) and elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in FRTL-5 thyroid cells. Cytoplasmic Ca^{2+} levels were measured using the fluorescent dye, indo-1. Monensin induced a dose-dependent increase in $[Ca^{2+}]_i$ in FRTL-5 cells. Inhibitors of intracellular Ca^{2+} release, TMB-8 and ryanodine, were unable to prevent the monensin effect on $[Ca^{2+}]_i$. The α_1-receptor antagonist, prazosin, did not block the monensin-stimulated increase in $[Ca^{2+}]_i$. In the absence of extracellular calcium there was a marked diminution in the monensin effect on $[Ca^{2+}]_i$, yet calcium channel antagonists (nifedipine, diltiazem and verapamil) did not inhibit the response. Replacement of Na^+ by choline chloride in the medium depressed the monensin-evoked rise in $[Ca^{2+}]_i$ by up to 84%. Furthermore, addition of the Na^+-channel agonist, veratridine, elicited an increase in $[Ca^{2+}]_i$, even though less dramatic than that caused by monensin. Ouabain increased the resting cytosolic Ca^{2+} concentration as well as the magnitude of the monensin effect on $[Ca^{2+}]_i$. The absence of any effect on the Na^+-ionophore evoked increase in $[Ca^{2+}]_i$ upon addition of tetrodotoxin (TTX) excluded a possible involvement of TTX-sensitive Na^+ channels. These data show that the rise in $[Ca^{2+}]_i$ induced by increasing $[Na^+]_i$ is largely dependent on both external Na^+ and Ca^{2+}. Calcium entry appears not to involve voltage-dependent or α_1-receptor sensitive Ca^{2+} channels, but may result from activation of an Na^+–Ca^{2+} exchange system.

Introduction

The key role of Ca^{2+} in stimulus-secretion coupling in secretory cells is well recognized. The intracellular free calcium concentration ($[Ca^{2+}]_i$) has been implicated as a mediator of the secretory response in the thyroid gland [1–6] as well as of thyroid hormone action in thymocytes [7]. A number of studies in different cell systems indicates also the importance of cytosolic Na^+ content in the control of $[Ca^{2+}]_i$ [8–18]. It has been suggested that a high cytoplasmic Na^+ concentration induces a rise in $[Ca^{2+}]_i$ through stimulated Ca^{2+} release from intracellular storage sites [8,10,11]. Other studies have indicated that a reversible Na^+–Ca^{2+} exchange activity in the cell membrane might be a factor regulating the cytosolic free calcium concentration [12–18]. Biological activities related to and/or controlled by the level of cytoplasmic Ca^{2+} are, therefore, linked to a sequence of events in which an increase in cytosolic Na^+ appears to be a significant step. The Na^+-selective ionophore, monensin [19–22] was used as a tool for the manipulation of cytoplasmic Na^+ levels. This carboxylic ionophore directly enhances the cytosolic Na^+ concentration by promoting an electrically neutral exchange of extracellular Na^+ for intracellular K^+ [19–21].

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The purpose of this study was to explore the role of $[Na^+]_i$ in changing the cytosolic free calcium concentration in rat FRTL-5 [23–30] thyroid cells and to define whether any elevation of intracellular free Ca^{2+} concentration is secondary to an increased influx of Ca^{2+} through cell membrane Ca^{2+} pathways and/or to a mobilization of Ca^{2+} from intracellular storage sites. The changes in cytosolic free calcium were evaluated by using the fluorescent dye, indo-1 [31,32]. These data show that monensin induced a dose-dependent increase in $[Ca^{2+}]_i$ which was due primarily to calcium influx and only in small part dependent on Ca^{2+} release from intracellular bound pools.

Materials and Methods

Cells

The FRTL-5 cells (ATCC CRL 8305) used in this study are a continuous strain of functioning epithelial cells derived from normal Fisher rat thyroids [23]. Their isolation, growth and basic characteristics have been described [24,25]. The strain used in this report, obtained from the Interthyr Institute Foundation (Baltimore, MD), was cloned from a culture of FRTL cells. These cells maintain the same differentiated features as FRTL cells but have been adapted to grow in a medium in which the content of serum has been shifted from 0.5% to 5%. The properties of FRTL-5 cell strain ATCC CRL 8305 have been described extensively [1–4,23–30].

The cells were grown under 5% CO_2/95% air at 37 °C, in Coon's modified Ham's F-12 medium enriched with 5% calf serum, 1 mM non-essential amino acids and six supplements (6 H), namely TSH (1 mU/ml), insulin (10 μg/ml), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (GHL, 2 ng/ml), somatostatin (10 ng/ml) and cortisol (1 nmol/l). Cells were plated in 100 × 20 mm culture dishes at a density of $2 \cdot 10^5$ cells/ml, fed twice weekly and passaged every 7–8 days using a trypsin-collagenase mixture [24–25]; the same mixture was also used to obtain separation of the cells for analysis. To avoid problems related to aging phenomena and mutations, we used cells only until their thirtieth passage.

Loading of the cells with indo-1

A suspension of the cells was prepared using a collagenase/trypsin/chick serum mixture [24,25] as for the usual passage. Cells were then collected by centrifugation (1000 rpm), washed with and suspended in the growth medium (6 H). This cell suspension was incubated for 1 h in a slow shaking water bath at 37 °C. After incubation, cells were centrifuged (1000 rpm), washed twice with 15 mM Hepes-buffered (pH 7.3) Hanks' balanced salt solution with Ca^{2+} and Mg^{2+} [$HBSS^{(+)}$] and resuspended in the same solution at a concentration of $5 \cdot 10^6$ to $5 \cdot 10^7$ cells/ml. The acetoxymethylester of indo-1 was added to this cell suspension at a final concentration of 5 μM. After 1 h incubation in a slow shaking water bath at 37 °C, cells were again washed twice with $HBSS^{(+)}$ containing 0.02% BSA and resuspended in the same solution to a concentration of $2.5 \cdot 10^6$ cells/ml. Aliquots were then transferred to a 3 ml quartz cuvette for each individual measurement.

Determination of fluorescence in the absence of extracellular calcium was performed using a Ca^{2+}-Mg^+ free HBSS [$HBSS^{(-)}$] containing 0.02% BSA in the final two washings and resuspending the cells in the same Ca^{2+} free solution. The cells were studied promptly to minimize depletion of internal calcium stores.

For measurements in the absence of extracellular Na^+, $HBSS^{(+)}$ was used in which Na^+ was replaced by equimolar amounts of choline chloride and the pH was adjusted to 7.3 in the final two washings after the cells were loaded with indo-1. The cells were then resuspended in the same Na^+-free solution for the fluorescence analysis.

Fluorescence measurements

All fluorescence recordings were performed by an SLM 8000 spectrofluorometer (SLM Instruments, Urbana, IL) or a PTI-deltascan spectrofluorometer (Photon, South Brunswick, NJ) both of which were connected to a personal computer for data storage and manipulation. The thermostatic cuvette holder in both instruments was equipped with a magnetic stirrer and a closed circulating water bath through which a constant temperature of 37 °C was maintained. The fluorescence signal was monitored using a slow-kinetic type of acquisition. The excitation wavelength was 350 nm in both instruments. The indo-1 emission was monitored as the ratio of intensities recorded at 405/480 nm when using the SLM-8000 and 395/480 nm when using the PTI-deltascan. Both excitation and emission spectra were recorded with 4 nm slit widths on the SLM-8000, while on the PTI-deltascan the excitation and emission wavelength bandpasses were set at 3 nm and 4 nm, respectively. Samples were placed in the cuvette holder 2–3 min before the beginning of each recording and resting Ca^{2+} levels were recorded for an additional 1–2 min, then appropriate reagents (monensin and others) were added and the signal was monitored as a function of time. Determinations of cytosolic Ca^{2+} concentration were calculated from the fluorescence ratio (R) obtained according to the following formula [31]:

$$[Ca^{2+}]_i = K_d \frac{(R - R_{min})(S_{f2})}{(R_{max} - R)(S_{b2})}$$

R_{min} is the ratio at zero calcium, R_{max} is the ratio at saturating calcium, K_d represents the dissociation constant for indo-1 in the appropriate conditions, S_f is the

fluorescence intensity of the free dye measured at 480 nm and S_b is the fluorescence intensity in saturating Ca^{2+} solution at 480 nm.

R_{min}, R_{max} and S_{f2}/S_{b2} were obtained following a method previously described [31]. While Triton was routinely used to obtain the R_{max}, similar results were observed using ionomycin (1–4 μM). According to this method, the emission spectra of indo-1 was measured as a function of free Ca^{2+}. Emission scan was the type of acquisition; excitation and emission slit widths were set to the values previously mentioned for the two instruments. A typical set of calibration constants obtained were $R_{max} = 3.52$, $R_{min} = 0.06$ and $S_{f_2}/S_{b_2} = 2.3$, values that agreed with the data reported in the above mentioned study [31]. The published value of 250 nmol/l was used as the dissociation constant (K_d) for calcium [31,32]. Resting calcium level values reported in this study and obtained from the SLM-8000 spectrofluorometer were corrected for cell autofluorescence, which accounted for $14 \pm 2\%$ ($n = 14$) of the total fluorescence. Measurements obtained with the PTI-Deltascan were automatically corrected for cell autofluorescence by the use of the pertinent software function. Viability of the cells after approx. 3 h fluorescence analysis was evaluated by Trypan blue exclusion and appeared to be between 90 – 96%.

Materials

Monensin, ionomycin, diltiazem, verapamil, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), ouabain, veratridine, tetrodotoxin (TTX) in sodium citrate buffer, choline chloride, Triton X-100, and EGTA were all purchased from Sigma (St. Louis, MO). Indo-1 acetoxymethyl (AM) ester was purchased from Molecular Probes, (Eugene, OR). Indo-1 pentasodium salt, ryanodine and nifedipine were obtained from Calbiochem (La Jolla, CA). Prazosin was a gift from Pfizer Laboratories (New York, NY). BAY K8644 was a gift of Dr. A. Scriabine (Miles, Pharmaceutical Div, West Haven, CT). All other chemicals used were of the purest grade commercially available.

Results

The basal concentration of cytoplasmic free Ca^{2+} of FRTL-5 thyroid cells loaded with indo-1/AM was estimated to be 182 ± 5 nM and it represents the mean $\pm$ S.E. of 115 measurements obtained from 15 different cell preparations.

Monensin increased the cytosolic Ca^{2+} concentration in a dose-dependent manner (Fig. 1). Monensin (10^{-4} M) induced an acute rise in $[Ca^{2+}]_i$ over baseline. The lowest effective concentration ($0.5 \cdot 10^{-8}$ M) caused a $22 \pm 4\%$ increase in cytosolic free Ca^{2+} levels. The highest concentration used caused an immediate increase in cytosolic free Ca^{2+} concentration which was

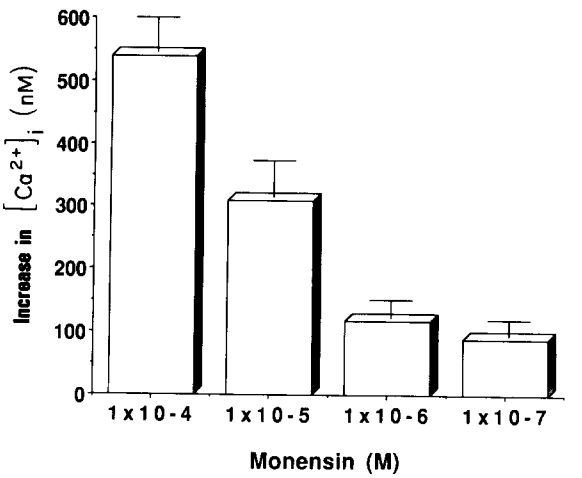

Fig. 1. Dose-dependent increase in $[Ca^{2+}]_i$ of FRTL-5 thyroid cells stimulated by the Na^+-ionophore, monensin. Data are expressed as nM increases of cytosolic Ca^{2+}. Values are the mean $\pm$ S.E. of 3–27 determinations at the different concentrations used, performed on various study days. Basal $[Ca^{2+}]_i = 182 \pm 5$ nM (mean $\pm$ S.E.; $n = 115$).

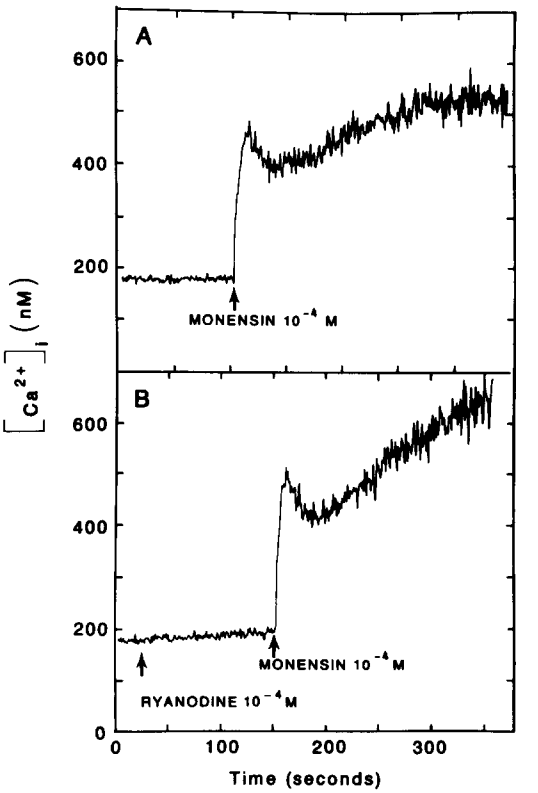

Fig. 2. Effect of blockers of intracellular Ca^{2+} release on elevation of $[Ca^{2+}]_i$ induced by monensin in FRTL-5 cells. Cells were loaded with indo-1/AM as described in Materials and Methods. (A) Trace represents the fluorescence signal of a control cell suspension. (B) Trace is the signal of cells in which ryanodine was added before the addition of monensin. The figure is representative of 12 analyses with a range of concentrations of ryanodine from 10^{-4} to 10^{-6} M. At least three different cell preparations were used.

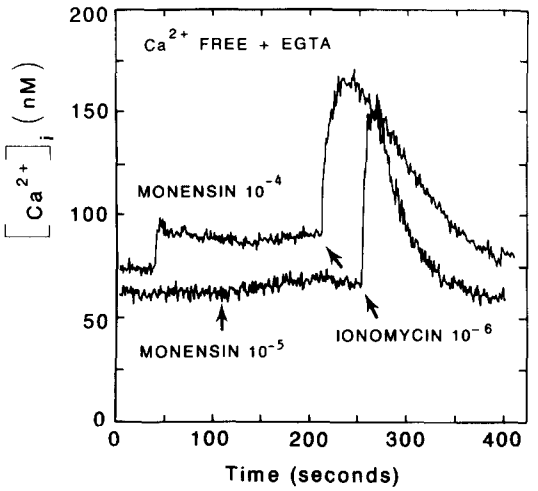

Fig. 3. Effect of monensin is largely dependent on extracellular Ca^{2+}. Cells were loaded with indo-1/AM and suspended in a Ca^{2+}-free medium (see Materials and Methods). EGTA (1 mM) was added at the beginning of each measurement. The figure is representative of at least 12 measurements performed on five different study days.

lost at lower doses of the ionophore. The maximal rise in $[Ca^{2+}]_i$ was obtained between 2–3 min after the addition of monensin and was steadily sustained over time. Monensin was dissolved in 95% ethanol, thus all samples included up to 1% (v/v, final concentration) ethanol. Control studies adding 1% ethanol alone to loaded FRTL-5 cells had no significant effect on the fluorescence signal (data not shown).

Increased $[Ca^{2+}]_i$ induced by monensin in FRTL-5 cells may be the result of Ca^{2+} release from intracellular stores or enhanced Ca^{2+} entry. To investigate the possible source of the cytosolic free Ca^{2+}, the Ca^{2+} store blockers TMB-8 [33] and ryanodine were used. The addition of TMB-8 at concentrations from 10^{-4} to 10^{-6} M, as well as the addition of ryanodine at 10^{-4} to 10^{-6} M (Fig. 2) had no effect on the monensin-stimulated rise in $[Ca^{2+}]_i$. Of note, at 10^{-4} M, TMB-8 alone paradoxically increased $[Ca^{2+}]_i$. From these results, a release of calcium ions from an intracellular source did not appear to contribute significantly to the rise in cytosolic Ca^{2+} observed upon the addition of monensin.

Further evidence to support this observation was obtained by studies conducted in a nominally Ca^{2+} free medium. The traces in Fig. 3 illustrate, as an example, a series of measurements in which cells were suspended in a Ca^{2+}–Mg^{+}-free HBSS. Monensin (10^{-4} M) elicited a small ($16 \pm 4\%$, $n = 16$) increase in the fluorescent signal which was lost at lower concentrations, whereas the addition of 1 μM ionomycin produced a prompt and transient increase in $[Ca^{2+}]_i$.

To determine whether Ca^{2+} channels were involved in the Ca^{2+} translocation through the plasma membrane, cells were exposed to various Ca^{2+} channel

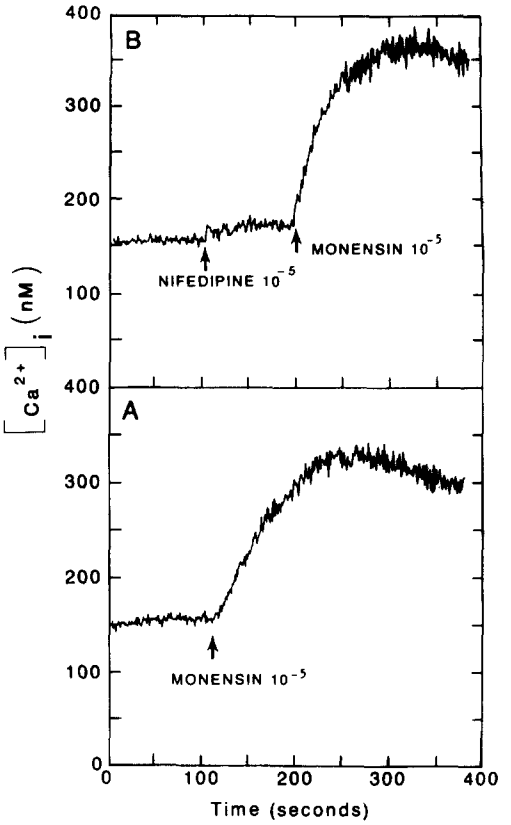

Fig. 4. Effect of a Ca^{2+}-channel antagonist on the monensin-stimulated increase in $[Ca^{2+}]_i$ in FRTL-5 cells. (A) The trace represents a control cell suspension. (B) The trace represents a cell suspension pretreated with the Ca^{2+}-channel antagonist, nifedipine. The lack of an inhibitory effect on the monensin-induced changes in $[Ca^{2+}]_i$ in FRTL-5 cells observed with nifedipine is shared by the other voltage-sensitive Ca^{2+} channel blockers studied (see Results). 3–12 measurements for each agent analyzed were performed, and different cell preparations were used with similar results.

TABLE I

Effect of prazosin on the monensin-stimulated increase in $[Ca^{2+}]_i$ in FRTL-5 thyroid cells

Prazosin was added to the FRTL-5 cell suspension between 1–2 min prior to the addition of monensin. Three control measurements were carried out to test the effect of prazosin 10^{-8} M on the NE-induced increase in $[Ca^{2+}]_i$. The results (not shown) indicated a complete inhibition of the NE effect on cytosolic Ca^{2+} in FRTL-5 cells, in agreement with data previously published [2]. Data reported in the table are mean ± S.E. of 3–5 determinations. Prazosin (10^{-7} M) was also used, but caused a significant, non-defined increase in fluorescence signal over the baseline at this concentration (data not shown).

Monensin concn. (M)	nM (%) increase in $[Ca^{2+}]_i$	Monensin and prazosin concn. (M)	nM (%) increase in $[Ca^{2+}]_i$
$1 \cdot 10^{-4}$	446 ± 158 (200%)	monensin 10^{-4} prazosin 10^{-8}	381 ± 194 (117%)
$1 \cdot 10^{-5}$	231 ± 80 (92%)	monensin 10^{-5} prazosin 10^{-8}	290 ± 102 (90%)
$1 \cdot 10^{-6}$	140 ± 15 (47%)	monensin 10^{-6} prazosin 10^{-8}	128 ± 13 (41%)

TABLE II

Effect of ouabain on $[Ca^{2+}]_i$ in FRTL-5 cells

Pretreating the cells with ouabain from 30 min to 1 h and 20 min induced an increase in $[Ca^{2+}]_i$ per se as well as an additive effect with monensin on cytosolic Ca^{2+} concentration. Control measurements were performed at time 0 to 30 min and after 1 h and 20 min. Data are the mean ± S.E. of 3 to 7 determinations obtained from 2 different cell preparations. Preincubation of the cell suspension with ouabain for 10 min had no effect on $[Ca^{2+}]_i$ (data not shown). n.d. = not determined.

	Resting $[Ca^{2+}]_i$ (nM)	nM (%) increase in $[Ca^{2+}]_i$ with monensin 10^{-4} M	nM (%) increase in $[Ca^{2+}]_i$ with monensin 10^{-5} M
Controls	243 ± 13	1205 ± 291 (396%)	501 ± 36 (106%)
Ouabain (10^{-3} M)	363 ± 63	2250 ± 739 (520%)	n.d.
Ouabain (10^{-4} M)	359 ± 8	n.d.	1139 ± 101 (217%)

antagonists [33,34] prior to the addition of monensin. In Fig. 4, the cell suspension was pretreated with nifedipine, following which the addition of monensin induced a rise in cytosolic Ca^{2+} concentration which was not influenced by the Ca^{2+} channel blocker. The data shown are representative of the effect obtained with all the concentrations of nifedipine tested (10^{-4} to 10^{-8} M). A significant difference could not be detected between control and treated FRTL-5 cell suspensions with either of the other voltage-sensitive Ca^{2+} channel blockers studied (verapamil and diltiazem) both at 10^{-4} to 10^{-8} M (data not shown). Neither the addition of the dihydropyridine agonist BAY K8644 (10^{-3} to 10^{-6} M) nor an increase of extracellular K^+ (20–80 mM) to depolarize the cell membrane, were able to produce a rise in $[Ca^{2+}]_i$ (data not shown).

α_1-adrenergic receptor stimulation is known to increase $[Ca^{2+}]_i$ in FRTL-5 cells [2]. To investigate the possible contribution of this pathway, the effect of the

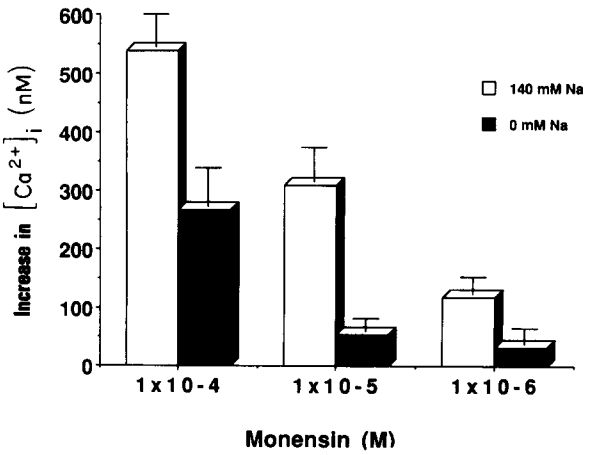

Fig. 5. Dose-dependent increase in cytosolic free Ca^{2+} concentration induced by monensin in the presence or absence of extracellular Na^+. Cells were loaded with indo-1/AM and suspended in a Na^+-free medium as described in Materials and Methods. In the figure are compared the increase in values of $[Ca^{2+}]_i$ obtained upon addition of the Na^+-ionophore to FRTL-5 cells in the presence (140 mM) and in the absence (0 mM) of extracellular Na^+. Data are the mean ± S.E. of 5–27 determinations performed on various study days.

α_1-blocker, prazosin (10^{-7} to 10^{-8} M) was tested. As illustrated in Table I, prior exposure of cells to prazosin did not affect the monensin-stimulated rise in $[Ca^{2+}]_i$, although from control studies, prazosin completely inhibited the effect of norepinephrine on the cytosolic Ca^{2+} concentration.

Different approaches were tried to establish the role of Na^+ in the monensin-mediated rise in $[Ca^{2+}]_i$ in FRTL-5 cells. As shown in Fig. 5, when Na^+ in the medium was replaced by choline chloride, a consistent reduction of the monensin-caused elevation of $[Ca^{2+}]_i$ could be observed.

Ouabain (10^{-3} and 10^{-4} M), an Na^+/K^+-ATPase inhibitor which increases the intracellular Na^+ concentration, caused an increase in the $[Ca^{2+}]_i$ per se and had an additive effect on the changes in $[Ca^{2+}]_i$ induced by monensin (10^{-4} to 10^{-5} M) in FRTL-5 cells (Table II).

As expressed in Table III, when the Na^+ channel agonist veratridine (10^{-4} or 10^{-5} M) was added, a 52 ± 6% and 28 ± 7% increase in $[Ca^{2+}]_i$ was observed, respectively. On a molar basis, veratridine was approx. 6-times less efficacious than monensin in elevating cytosolic Ca^{2+} levels. The magnitude of the percentage increase in $[Ca^{2+}]_i$ induced by veratridine (10^{-3} M)

TABLE III

Veratridine effect on cytosolic free Ca^{2+} concentrations in FRTL-5 thyroid cells

The results shown are the mean ± S.E. of 4–7 measurements obtained from four different cell preparations. Data are expressed as nM (%) increase of the Ca^{2+} level over the baseline.

Agents concn. (M)	nM (%) increase in $[Ca^{2+}]_i$
Monensin	
$1 \cdot 10^{-4}$	583 ± 126 (362%)
$1 \cdot 10^{-5}$	255 ± 31 (142%)
Veratridine	
$1 \cdot 10^{-4}$	97 ± 10 (52%)
$1 \cdot 10^{-5}$	60 ± 12 (28%)

TABLE IV

Monensin-stimulated increase in $[Ca^{2+}]_i$ in FRTL-5 thyroid cells pretreated with tetrodotoxin (TTX)

Tetrodotoxin was added to the cuvette about 1 min before the addition of monensin. Data represent the mean ± S.E. of three or four determinations obtained from three different cell preparations.

Monensin conc. (M)	nM (%) increase in $[Ca^{2+}]_i$	TTX and monensin concn. (M)	nM (%) increase in $[Ca^{2+}]_i$
$1 \cdot 10^{-4}$	343 ± 35 (121%)	TTX $4.7 \cdot 10^{-6}$ Mon. $1 \cdot 10^{-4}$	394 ± 80 (157%)
$1 \cdot 10^{-5}$	218 ± 41 (79%)	TTX $4.7 \cdot 10^{-6}$ Mon. $1 \cdot 10^{-5}$	192 ± 44 (79%)
$1 \cdot 10^{-6}$	142 ± 13 (51%)	TTX $4.7 \cdot 10^{-6}$ Mon. $1 \cdot 10^{-6}$	142 ± 38 (53%)

reached values of 1065 ± 405% ($n = 5$). However, under such high concentrations veratridine appeared to have a toxic effect as ascertained by Trypan blue exclusion (viability about 50%).

Pretreatment of cells with tetrodotoxin did not inhibit the monensin changes in $[Ca^{2+}]_i$ as illustrated in Table IV. The results suggest the monensin-mediated effects on $[Ca^{2+}]_i$ in FRTL-5 cells do not involve TTX-sensitive sodium channels.

Discussion

The observations derived from the data presented in this study implicate a relationship between Na^+ entry in FRTL-5 thyroid cells and a rise in cytosolic Ca^{2+} concentration recorded with the fluorescent probe, indo-1. These data show that the Na^+-specific ionophore, monensin, which increases $[Na^+]_i$, elevated $[Ca^{2+}]_i$ in a dose-dependent manner.

Maintenance of a physiologic cytoplasmic free Ca^{2+} concentration is the result of an appropriately combined interplay between the Ca^{2+} devices present at the cell membrane level and the several organelles and Ca^{2+}-binding proteins present within the cell. Unlike some other cell systems [8–11], the mechanism by which an increase in Na^+ influx is coupled to an elevation of cytosolic free Ca^{2+} in FRTL-5 cells appears to involve only a minimal calcium release from intracellular sources. The absence of an inhibitory effect on the monensin stimulated rise of $[Ca^{2+}]_i$ by agents which block the release and mobilization of calcium from intracellular storage sites (e.g., ryanodine and TMB-8) suggests that the augmented cytosolic free calcium ions observed upon addition of monensin are predominantly of extracellular origin. Additional evidence supporting this conclusion is the observation that the monensin effect is almost completely abolished by removal of external Ca^2 (Fig. 3). In contrast, subsequent addition of the Ca^{2+}-specific ionophore, ionomycin, to FRTL-5 cells suspended in a nominally free Ca^{2+} medium induced a transient increase in $[Ca^{2+}]_i$ due to the known ability of ionomycin to release calcium sequestered within the cell [35]. Therefore, the translocation of Ca^{2+} ions into the cell from the outside accounts for the initial as well as the sustained phase of the increased $[Ca^{2+}]_i$ observed in cells treated with monensin. From the above results, the cell membrane appears to be the primary target of the indirect monensin action on increasing cytosolic Ca^{2+} in FRTL-5 thyroid cells.

Active movement of Ca^{2+} ions across the plasma membrane of cells is accomplished by a complex interplay of voltage potential and receptor regulated Ca^{2+} channels, an Na^+–Ca^{2+} exchange system and ATP energized Ca^{2+} pumps. The balanced interplay between those different pathways present in the cell membrane is a determinant in regulating cell calcium homeostasis [36].

Taking into account the above considerations, the possible involvement of slow calcium channels as a plausible pathway of Ca^{2+} entry was investigated. It is reported that the major influx of Na^+ caused by monensin is coupled to a K^+ and H^+ efflux, with consequent membrane potential changes and an increase in intracellular pH [19]; therefore, Ca^{2+} channels operating through membrane potential changes might be involved in the Na^+-mediated effect on $[Ca^{2+}]_i$. However, the inability of diltiazem, nifedipine and verapamil to inhibit the increase in $[Ca^{2+}]_i$ produced by monensin suggests that Ca^{2+} entry does not occur through voltage-sensitive Ca^{2+} channels. Moreover, the lack of effect of BAY K8644 or increased extracellular K^+ concentrations to enhance cytosolic Ca^{2+} may reflect a relative paucity or absence of voltage-sensitive Ca^{2+} channels in FRTL-5 cells. These data are consistent with the interpretation that the monensin effect on $[Ca^{2+}]_i$ is not due to the activation of voltage-sensitive Ca^{2+} channels.

Since α_1-adrenergic receptor activation has been found to be accompanied by increased $[Ca^{2+}]_i$ in FRTL-5 cells [2], receptor regulated Ca^{2+} channels could be an alternative site involved in the monensin effect on $[Ca^2]_i$. However, prazosin pretreatment failed to inhibit this effect. Such results exclude the α_1-receptor regulated Ca^{2+} channel as a possible mechanism for the monensin induced Ca^{2+} entry.

A third possibility would be the activation of a plasma membrane Na^+/Ca^{2+} exchanger. When intracellular Na^+ increases, this system transports Na^+ to the extracellular space in exchange for Ca^{2+} transport inward [12–18]. Such an event could explain the rise in $[Ca^{2+}]_i$ induced by monensin, as well as the substantial attenuation of this response in the absence of extracellular Na^+. Additional evidence that such a pathway might be involved is provided by the studies with veratridine. Veratridine increases $[Na^+]_i$ by a mechanism distinct

from that of monensin. Nevertheless, a prompt increase in cytosolic free calcium occurred. Moreover, in a Ca^{2+}-free buffer, veratridine did not elicit a change in $[Ca^{2+}]_i$ (data not shown), indicating that the source of $[Ca^{2+}]_i$ was derived from the extracellular milieu. The inability of tetrodotoxin to prevent the rise in $[Ca^{2+}]_i$ ruled out a possible involvement of the tetrodotoxin sensitive voltage-dependent Na^+ channels in the action of the Na^+-ionophore. Further support was generated in experiments using ouabain. When the Na^+/K^+-ATPase is inhibited, cellular Na^+ content increases and has been associated with a rise in cellular Ca^{2+} [16]. Ouabain pretreatment increased both the basal $[Ca^{2+}]_i$ and the increase after monensin, providing evidence that FRTL-5 cells possess a plasma membrane Na^+/Ca^{2+} exchanger which might be responsible for the increase in $[Ca^{2+}]_i$ induced by monensin.

As indicated earlier, the absence of extracellular Na^+ depressed the monensin induced increase in $[Ca^{2+}]_i$ by as much as 84% (Fig. 5). However, a modest monensin effect on $[Ca^{2+}]_i$ was still present in cells suspended in an Na^+-free buffer. According to this observation, a portion of the monensin mediated rise in cytosolic free Ca^{2+} concentration may be independent of Na^+ influx. Alternatively, since the basal $[Ca^{2+}]_i$ increased in Na^+ free buffer, presumably due to activation of the Na^+/Ca^{2+} exchanger, this efflux of Na^+ would then be available extracellularly for monensin to transport into the cell. It has been reported that monensin catalyzes Na^+–H^+ exchange leading to an increase in pH_i [19] and, from studies conducted in different cell systems, it has been found that a rise in intracellular pH significantly elevated the $[Ca^{2+}]_i$ [14,37]. Therefore, it seems reasonable to speculate a combined role of Na^+ and pH_i in the monensin effect on $[Ca^{2+}]_i$ in FRTL-5 cells. Further investigations are needed to define the exact role of each of the above components and the correct sequence of events triggering the observed augmentation of cytosolic free Ca^{2+} concentration in FRTL-5 thyroid cells for a better understanding of the physiologic conditions leading ultimately to hormone secretion.

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