Tunicamycin increases intracellular calcium levels in bovine aortic endothelial cells

BARBARA J. BUCKLEY AND A. R. WHORTON
Departments of Pharmacology and Medicine, Duke University Medical Center, Durham, North Carolina 27710

Buckley, Barbara J., and A. R. Whorton. Tunicamycin increases intracellular calcium levels in bovine aortic endothelial cells. Am. J. Physiol. 273 (Cell Physiol. 42): C1298–C1305, 1997.—Tunicamycin is a nucleoside antibiotic that inhibits protein glycosylation and palmitoylation. The therapeutic use of tunicamycin is limited in animals because of its toxic effects, particularly in cerebral vasculature. Tunicamycin decreases permeability of the endothelial isoform of nitric oxide synthase, stimulates nitric oxide synthesis, and increases the concentration of intracellular calcium ([Ca\(^{2+}\)]\(_i\)) in bovine aortic endothelial cells (B. J. Buckley and A. R. Whorton, FASEB J. 11: A110, 1997). In the present study, we investigated the mechanism by which tunicamycin alters [Ca\(^{2+}\)]\(_i\); using the Ca\(^{2+}\)-sensitive dye fura 2. We found that tunicamycin increased [Ca\(^{2+}\)]\(_i\) without increasing levels of inositol phosphates. When cells were incubated in the absence of extracellular Ca\(^{2+}\), [Ca\(^{2+}\)]\(_i\) rapidly rose in response to tunicamycin, although a full response was not achieved. The pool of intracellular Ca\(^{2+}\) mobilized by tunicamycin overlapped with that mobilized by thapsigargin. Extracellular nickel blocked a full response to tunicamycin when cells were incubated in the presence of extracellular Ca\(^{2+}\). The effects of tunicamycin on [Ca\(^{2+}\)]\(_i\) were partially reversed by washing out the drug, and the remainder of the response was inhibited by removing extracellular Ca\(^{2+}\). These results indicate that tunicamycin mobilizes Ca\(^{2+}\) from intracellular stores in a manner independent of phospholipase C activation and increases the influx of Ca\(^{2+}\) across the plasma membrane.

intracellular calcium stores; calcium influx; inositol trisphosphate; thapsigargin; palmitoylation

TUNICAMYCIN IS A NUCLEOSIDE antibiotic produced by Streptomyces lyosuperificus that inhibits cell wall polymer synthesis (7, 18). The therapeutic usefulness of tunicamycin has been limited by its toxicity in animals. Structurally similar analogs of tunicamycin, the corynebacterins, cause central nervous system disease in livestock (12). Tunicamycin disrupts cerebral microvessels, causing ischemia and hypoxic injury to the cerebellum in animal models (8). Tunicamycin is also hepatotoxic, producing a periportal pattern of damage, resulting in the denudation of hepatocytes into hepatic blood vessels and the formation of emboli in the pulmonary and cerebral capillary vasculature (9). Ultrastructural studies of hepatocytes and cerebral endothelium demonstrate marked dilation of rough endoplasmic reticulum cisternae in response to tunicamycin (9, 10). Increased permeability of brain microvessels and cultured endothelial cells has also been described after treatment with this agent (10, 21).

The mechanism underlying the toxicity of tunicamycin has not been well defined. However, tunicamycin is well known as an inhibitor of protein glycosylation. It blocks N-linked protein glycosylation by inhibiting UDP-N-acetylglucosamine-dolichyl-phosphate: N-acetylglucosamine-1-phosphate transferase (7, 16). These effects have been noted in many systems in vitro and typically involve several hours of treatment of cells with 0.05–5 µg/ml tunicamycin (7, 16). In addition, recent studies have demonstrated that tunicamycin inhibits protein palmitoylation in cells after several minutes of treatment with 1–25 µg/ml (16, 17). We recently found that tunicamycin decreased palmitoylation of the endothelial isoform of nitric oxide synthase and stimulated nitric oxide synthesis in bovine aortic endothelial cells (4). Unexpectedly, tunicamycin also increased intracellular calcium levels ([Ca\(^{2+}\)]\(_i\)) in a concentration-dependent manner (4).

In the present study, we have investigated the mechanism by which tunicamycin alters [Ca\(^{2+}\)]\(_i\) in bovine aortic endothelial cells. These experiments were conducted by spectrofluorometric methods, using the Ca\(^{2+}\)-sensitive dye fura 2. Our results demonstrate that tunicamycin mobilizes intracellular Ca\(^{2+}\) stores and increases the influx of Ca\(^{2+}\) across the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials. Fetal bovine serum was from HyClone (Logan, UT). Tissue culture plasticware was from NUNC (Fisher Scientific, Raleigh, NC). Myo-[2-\(^{3}H\)]inositol and D-[2-\(^{3}H\)]inositol 1-phosphate, inositol 1,4-bisphosphate, and inositol 1,4,5-triphosphate were obtained from DuPont NEN (Boston, MA). All other cell culture reagents were from Gibco (Grand Island, NY). Tunicamycin was from Boehringer Mannheim (Indianapolis, IN), and thapsigargin was from LC Services (Woburn, MA). The acetylmethyl ester of fura 2 (fura 2-AM) was obtained from Molecular Probes (Eugene, OR). Anion exchange resin AG1X8, 100–200 mesh, formate form, was from Bio-Rad (Hercules, CA). All other reagents were from Sigma Chemical (St. Louis, MO).

Cell isolation and culture. Endothelial cells were isolated and cultured by established methods as previously described (3, 6). Briefly, bovine aortic segments were cleaned and opened, and the endothelium was removed by scraping. Isolates were plated on collagen-coated tissue culture plasticware or on glass coverslips (1.25 × 3 cm). Cells were incubated at 37°C in an atmosphere of 7.5% CO\(_2\) in air in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (vol/vol) fetal bovine serum and antibiotic-antimycotic (100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, and 250 ng/ml amphotericin B). Cells cultured on plastic were passaged by treatment with trypsin-EDTA. Endothelial cells exhibited typical cobblestone appearance and were shown to express factor VIII-related antigen and to incorporate 1,1’dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate-labeled low-density lipoprotein specifically.
Use of tunicamycin. Tunicamycin was handled with great care because of its extreme toxicity. After dissolution in 10 mM NaOH, the concentration of tunicamycin was established spectrophotometrically, based on an absorbance at 260 nM of 0.120 for a 10 µg/ml solution (16, 17).

Measurement of [Ca\(^{2+}\)]\(_i\). To monitor changes in [Ca\(^{2+}\)]\(_i\), confluent monolayers of cells on glass coverslips were incubated for 1 h at 37°C with 20 µM fura 2-AM in Hanks’ balanced salt solution containing 10 mM N-2-hydroxethylpiperazine-N’-2-ethanesulfonic acid (HHBSS) containing 0.25% bovine serum albumin (BSA), pH 7.4, as previously described (4, 6). Briefly, fura 2-loaded cells were placed across the diagonal in a 3-ml cuvette fitted with inlet and outlet lines. The cuvette was perfused at 3 ml/min with HHBSS in the absence of BSA, pH 7.4, and maintained at 37°C. Agonists were added at the final concentration by stopping flow and rapidly exchanging the contents of the cuvette with agonist-containing buffer, using a 20-ml syringe. After 1 min, flow was restored. Tunicamycin (10 mM NaOH) and thapsigargin (in dimethyl sulfoxide) were diluted in perfusate immediately before addition to cells. The ratio of fluorescence at emission wavelengths of 510 nM of cells alternately excited at 350 and 385 nm was continuously monitored (RatioMaster Spectrofluorometer; PTI, South Brunswick, NJ).

Analysis of inositol phosphates. Measurement of inositol phosphate production was carried out as previously described (6). Subconfluent cells in well plates were incubated with 10 µCi/ml [3H(N)]inositol for 24 h in 2 ml DMEM containing 5% fetal bovine serum and 1% antibiotic-antimycotic. After reaching confluence, cells were washed free of culture medium and incubated at 37°C in HHBSS, pH 7.4, containing 10 mM LiCl for 15 min. Cells were stimulated with bradykinin, tunicamycin, or vehicle (10 mM NaOH) in HHBSS containing LiCl for the indicated times. The reaction was stopped by rapidly removing the buffer, washing twice with HHBSS-LiCl, and adding 1 ml ice-cold methanol. The cells were scraped from the flasks, and the flasks were rinsed with an additional 0.8 ml of cold water. Chloroform (1 ml) and methanol (1 ml) were added to the combined extracts, and the samples were sonicated. The extracts were treated with an additional 1 ml of chloroform and 0.8 ml of water and centrifuged. The upper aqueous phase containing the labeled inositol polyphosphates was removed and applied to a column containing 1-ml bed volume of anion exchange resin (AG1X8, 100–200 mesh, formate form). Free inositol and glycerol phosphate were eluted with 12 ml of water and 10 ml of a solution containing 5 mM sodium tetraborate and 60 mM ammonium formate. The columns were then treated with 8 ml each of 0.2, 0.4, and 1.0 mM ammonium formate in 0.1 M formic acid to elute inositol monophosphates, inositol bisphosphates, and inositol trisphosphates, respectively. Retention times on the anion exchange column were verified with the use of labeled standards.

Protein analysis. Protein mass was determined by a modified Lowry method, after trichloroacetic acid precipitation of detergent-containing samples (2).

RESULTS

We have recently reported a concentration-dependent increase in [Ca\(^{2+}\)]\(_i\) in endothelial cells treated with 5–20 µg/ml tunicamycin (4). These changes occurred within seconds to minutes after the addition of tunicamycin, with the most rapid responses occurring at concentrations of 15 and 20 µg/ml tunicamycin. The magnitude of the rise in [Ca\(^{2+}\)]\(_i\) was comparable to that obtained with maximal doses of thapsigargin, an agent that elevates [Ca\(^{2+}\)] in endothelial cells by mobilizing intracellular Ca\(^{2+}\) stores and activating Ca\(^{2+}\) influx (6, 21).

To investigate whether tunicamycin was acting on intracellular stores of Ca\(^{2+}\), we treated cells with tunicamycin in the presence and absence of extracellular Ca\(^{2+}\). This protocol involved perfusion with 12.5 µg/ml tunicamycin in the absence of BSA, which is known to sequester tunicamycin (16, 17). Endothelial cells in Ca\(^{2+}\)-containing HHBSS responded to tunicamycin with a large increase in [Ca\(^{2+}\)]\(_i\), after a lag period of several minutes (Fig. 1). The mechanism for this delayed response to tunicamycin is not known. When cells were rinsed with tunicamycin-free buffer, the effect of tunicamycin on [Ca\(^{2+}\)]\(_i\) was partially reversed. In contrast, endothelial cells in Ca\(^{2+}\)-free HHBSS containing 1 mM ethylene glycol-bis(β-aminomethyl ether)-N,N’,N”-tetraacctetic acid (EGTA) responded to tunicamycin with a rapid and multiphasic increase in [Ca\(^{2+}\)]\(_i\). The magnitude of the tunicamycin response was smaller and the duration of the tunicamycin response was shorter in the absence of extracellular Ca\(^{2+}\). In addition, the Ca\(^{2+}\) response occurred more quickly and consisted of more than one transient when Ca\(^{2+}\) was absent. These results suggest that tunicamycin mobilized intracellular Ca\(^{2+}\) stores.

Many agonists release intracellular Ca\(^{2+}\) from intracellular stores by activating the phospholipase C pathway and increasing levels of inositol 1,4,5-trisphosphate (15). To determine whether tunicamycin acted by this mechanism, we measured the formation of inositol phosphates.
phosphates in control endothelial cells and in those treated with 12.5 µg/ml tunicamycin. Bradykinin-treated cells were used as positive controls. Figure 2 demonstrates that tunicamycin failed to increase inositol phosphate levels after 2.5, 5, and 10 min of treatment, during which time \([\text{Ca}^{2+}]_i\) is highest. In contrast, inositol phosphate levels were increased two- to three-fold in bradykinin-treated cells. These results indicate that tunicamycin mobilized intracellular \(\text{Ca}^{2+}\) stores in a manner independent of phospholipase C activation.

The non-receptor agonist thapsigargin mobilizes intracellular \(\text{Ca}^{2+}\) stores by inhibiting \(\text{Ca}^{2+}\)-ATPase on the endoplasmic reticulum membrane, thus leading to the release of \(\text{Ca}^{2+}\) from intracellular stores (21). To determine whether tunicamycin acted on the same intracellular \(\text{Ca}^{2+}\) store as thapsigargin, we treated endothelial cells with tunicamycin alone or with thapsigargin followed by tunicamycin in the absence of extracellular \(\text{Ca}^{2+}\). A supramaximal concentration of thapsigargin (1 µM) was used in these studies to completely deplete stores of intracellular \(\text{Ca}^{2+}\) (6). It should be noted that no refilling of depleted stores can occur after thapsigargin treatment in the absence of extracellular \(\text{Ca}^{2+}\) (6). When cells were treated with tunicamycin (12.5 µg/ml), a rapid and multiphasic increase in \([\text{Ca}^{2+}]_i\) was observed (Fig. 3A). Tunicamycin-induced \(\text{Ca}^{2+}\) transients were complete within 10 min of incubation in \(\text{Ca}^{2+}\)-free buffer (Fig. 1 and data not shown). After \([\text{Ca}^{2+}]_i\) returned to baseline, \(\text{Ca}^{2+}\)-free buffer was rapidly replaced with \(\text{Ca}^{2+}\)-complete buffer. We observed a rapid and prolonged increase in \([\text{Ca}^{2+}]_i\), representing the influx of \(\text{Ca}^{2+}\) across the plasma membrane to refill the depleted intracellular stores (6, 19). When cells were treated with thapsigargin, a rapid increase in

![Fig. 2. Effect of agonists on inositol phosphate formation in bovine aortic endothelial cells. Subconfluent monolayers of cells were incubated with 10 µCi/ml myo-[2-3H(N)]inositol for 24 h as described in EXPERIMENTAL PROCEDURES. After reaching confluence, cells were rinsed extensively and incubated in HHBSS containing 10 mM LiCl. Cells were treated with 1 µM bradykinin for 0.5 min (●) or with 12.5 µg/ml TM for 2.5, 5, and 10 min (●) or with vehicle (vehicle for bradykinin is water (○), vehicle for TM is 10 mM NaOH (□)) in HHBSS containing 10 mM LiCl. Reaction was rapidly terminated, aqueous extracts of cell monolayers were prepared, and inositol phosphates were separated by anion exchange chromatography. A: inositol monophosphates. B: inositol bisphosphates. C: inositol trisphosphates. Data are given as means ± SE; n = 3 experiments. dpm, Disintegrations/min.]
followed by thapsigargin in the absence of extracellular Ca\(^{2+}\) (Fig. 4). Tunicamycin treatment (12.5 \(\mu\)g/ml) resulted in a rapid and multiphasic increase in [Ca\(^{2+}\)], as shown before, and subsequent treatment with thapsigargin (1 \(\mu\)M) also led to a rise in [Ca\(^{2+}\)] (Fig. 4A). The response to thapsigargin after tunicamycin treatment (Fig. 4A) was slightly smaller than that found in cells treated in thapsigargin alone (Fig. 4B). These results suggest that tunicamycin only partially mobilized intracellular Ca\(^{2+}\) stores or that Ca\(^{2+}\) mobilized by tunicamycin was rapidly resequestered. When extracellular Ca\(^{2+}\) was restored, a similar increase in [Ca\(^{2+}\)] was observed in cells treated with both tunicamycin and thapsigargin and in cells treated with thapsigargin alone (Fig. 4, A and B). This result suggests that the degree of intracellular store depletion was similar under both

Fig. 3. Effect of thapsigargin (TG) on TM-stimulated Ca\(^{2+}\) mobilization. Cells were superfused with Ca\(^{2+}\)-free HHBSS containing 1 mM EGTA (EGTA). At indicated times, a rapid exchange with buffer containing 1 \(\mu\)M TG or 12.5 \(\mu\)g/ml TM was made. Superfusion was continued for 5 min, followed by a rapid rinse with EGTA-HHBSS. At end of experiment, EGTA-HHBSS was rapidly replaced with Ca\(^{2+}\)-complete HHBSS (Ca\(^{2+}\)). A: cells were exposed to TM. B: cells were exposed to TG followed by TM. Data are given as means \(\pm\) SE; \(n = 3\) experiments.

[Ca\(^{2+}\)], was observed that returned to baseline or lower levels after several minutes (Fig. 3B). No further increase in [Ca\(^{2+}\)] was seen when these cells were subsequently treated with tunicamycin, indicating that tunicamycin acted on the stores depleted by thapsigargin. When extracellular Ca\(^{2+}\) was restored, a large and prolonged increase in [Ca\(^{2+}\)] occurred, representing capacitative Ca\(^{2+}\) influx and repletion of internal Ca\(^{2+}\) stores. The magnitude of this latter increase was smaller in cells treated only with tunicamycin (Fig. 3A) than in cells treated with thapsigargin and tunicamycin (Fig. 3B). This result suggests that intracellular Ca\(^{2+}\) stores were only partially emptied during tunicamycin treatment or that the Ca\(^{2+}\) mobilized by tunicamycin was resequestered.

In another set of experiments, endothelial cells were treated with thapsigargin alone or with tunicamycin

Fig. 4. Effect of TM on TG-stimulated Ca\(^{2+}\) mobilization. Cells were superfused with Ca\(^{2+}\)-free HHBSS containing 1 mM EGTA (EGTA). At indicated times, a rapid exchange with buffer containing 12.5 \(\mu\)g/ml TM or 1 \(\mu\)M TG was made. Superfusion was continued for 5 min, followed by a rapid rinse with EGTA-HHBSS. At end of experiment, EGTA-HHBSS was rapidly replaced with Ca\(^{2+}\)-complete HHBSS (Ca\(^{2+}\)). A: cells were exposed to TM followed by TG. B: cells were exposed to only TG. Data are given as means \(\pm\) SE; \(n = 3\) experiments.
sets of experimental conditions. Furthermore, these findings are consistent with other data showing that intracellular store depletion by tunicamycin was small and/or transient, since restoration of extracellular Ca\(^{2+}\) after tunicamycin treatment led to only a small rise in [Ca\(^{2+}\)], (Fig. 3A). Taken together, these studies provide evidence that tunicamycin acted on pools of intracellular Ca\(^{2+}\) that are sensitive to thapsigargin and that the Ca\(^{2+}\) mobilized by tunicamycin was resequestered in the absence of extracellular Ca\(^{2+}\).

Results from Fig. 1 suggested that the large and sustained response to tunicamycin required the influx of Ca\(^{2+}\) across the plasma membrane. To investigate the role of Ca\(^{2+}\) influx in the tunicamycin response, we tested the effects of removal of extracellular Ca\(^{2+}\) on the tunicamycin response while [Ca\(^{2+}\)], was high. Figure 5 demonstrates the results of this experiment. Endothelial cells were treated with 12.5 µg/ml tunicamycin in Ca\(^{2+}\)-complete buffer. After a maximal response was obtained, tunicamycin was rapidly rinsed out of the system and a small decrease in [Ca\(^{2+}\)], was observed. Next, the Ca\(^{2+}\)-complete buffer was rapidly replaced with Ca\(^{2+}\)-free buffer containing 1 mM EGTA. We observed an attenuation of the response, with [Ca\(^{2+}\)], returning to baseline or slightly lower. These data indicate that the prolonged increase in [Ca\(^{2+}\)], after tunicamycin was due to the influx of extracellular Ca\(^{2+}\).

In addition, the Ca\(^{2+}\) entry mechanism activated by tunicamycin remained active after removal of tunicamycin and only slowly decayed when extracellular Ca\(^{2+}\) was removed. In a second experiment, we tested the effects of restoring extracellular Ca\(^{2+}\) after treatment with tunicamycin in the absence of extracellular Ca\(^{2+}\). Endothelial cells were treated with 12.5 µg/ml tunicamycin in Ca\(^{2+}\)-free HHBSS containing 1 mM EGTA. After [Ca\(^{2+}\)] returned to baseline, EGTA-HHBSS was rapidly replaced with Ca\(^{2+}\)-complete buffer. We observed a rapid and prolonged increase in [Ca\(^{2+}\)], indicating that capacitative Ca\(^{2+}\) entry was initiated after depletion of intracellular Ca\(^{2+}\) stores (Fig. 6). These results provide evidence that Ca\(^{2+}\) entry was active after tunicamycin was removed from the system. In a third study, we treated cells with agonists in the presence and absence of extracellular nickel, which blocks the influx of extracellular Ca\(^{2+}\). Experiments with bradykinin (1 µM) and thapsigargin (1 µM) showed that 5 mM NiCl\(_2\) dramatically reduced the increase in [Ca\(^{2+}\)], presumably due to influx of Ca\(^{2+}\) across the plasma membrane. In the case of tunicamycin (10 µg/ml), the increase in [Ca\(^{2+}\)] observed was smaller in magnitude and significantly delayed in the presence of nickel compared with the response in the absence of nickel (Fig. 7C). These results suggest that a large component of the tunicamycin response was due to the influx of Ca\(^{2+}\) across the plasma membrane.

**DISCUSSION**

These studies clearly show that tunicamycin increased [Ca\(^{2+}\)], and provide several insights into the mechanisms involved. First, tunicamycin mobilized intracellular Ca\(^{2+}\) stores without phospholipase C activation and the generation of inositol trisphosphate. The pool of intracellular Ca\(^{2+}\) mobilized by tunicamycin was also mobilized by thapsigargin. Unlike those of thapsigargin, the effects of tunicamycin were probably not due to inhibition of Ca\(^{2+}\)-ATPase. We found no evidence that tunicamycin inhibited endoplasmic reticulum Ca\(^{2+}\)-ATPase, since partial refilling of the intracellular stores occurred in tunicamycin-treated cells in the absence of extracellular Ca\(^{2+}\) (Figs. 3A and 4A). Inhibition of plasma membrane Ca\(^{2+}\)-ATPase by tunicamycin was also unlikely, since [Ca\(^{2+}\)], rapidly returned to basal in tunicamycin-treated cells after removal of extracellular Ca\(^{2+}\) (Fig. 5). In addition, the effects of tunicamycin were more rapidly reversible than those of thapsigarin-
In thapsigargin-treated cells, \([\text{Ca}^{2+}]_i\) slowly returned to baseline over several hours after the removal of thapsigargin (data not shown). However in tunicamycin-treated cells, \([\text{Ca}^{2+}]_i\) partially returned to baseline during a 15-min rinse period and increased in response to a second treatment with tunicamycin (data not shown).

In addition to mobilizing intracellular \(\text{Ca}^{2+}\) stores, tunicamycin stimulated the influx of \(\text{Ca}^{2+}\) across the plasma membrane. Several lines of evidence support this conclusion. First, a full response to tunicamycin required extracellular \(\text{Ca}^{2+}\) (Fig. 1). Second, the large increase in \([\text{Ca}^{2+}]_i\) after tunicamycin treatment was quickly reversed by the addition of \(\text{Ca}^{2+}\)-free EGTA buffer (Fig. 5). Third, extracellular nickel blocked \(\text{Ca}^{2+}\) influx in response to bradykinin and thapsigargin and resulted in a delayed and attenuated response to tunicamycin (Fig. 7). These results suggest that tunicamycin activated an influx mechanism. Although the mechanism for \(\text{Ca}^{2+}\) influx is not known, influx probably occurred by capacitative entry after depletion of internal stores (19).

In addition to its immediate effects on \(\text{Ca}^{2+}\) entry, tunicamycin also led to sustained activation of the influx pathway. After removal of tunicamycin from the system, influx remained active (Figs. 5 and 6). These results suggest either that significant amounts of tunicamycin remained associated with the cells or that tunicamycin had effects that persisted after the rinse. Tunicamycin has a complex structure, including a fatty acyl moiety that would have enhanced its association with membranes. In addition, tunicamycin has a nucleoside structure that may have increased membrane permeability to \(\text{Ca}^{2+}\). In that case, significant amounts of tunicamycin remaining in the membrane after the rinse may have led to a continued increase in \(\text{Ca}^{2+}\) permeability. We observed partial refilling of tunicamycin-depleted intracellular stores in the absence of extracellular \(\text{Ca}^{2+}\), indicating that endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase was active (Figs. 3 and 4). However, the increased permeability may have resulted in a persistent partial depletion of intracellular \(\text{Ca}^{2+}\) pools and continued activation of capacitative \(\text{Ca}^{2+}\) entry. One would expect complete refilling of the intracellular stores in the presence of extracellular \(\text{Ca}^{2+}\) when tunicamycin was eventually cleared from the membrane, and, in fact, slow return of \([\text{Ca}^{2+}]_i\) toward baseline was observed after the removal of tunicamycin from the buffer. Alternatively, tunicamycin may have altered the activity of specific proteins involved in \(\text{Ca}^{2+}\) homeostasis, resulting in persistent effects. Tunicamycin is a well-known inhibitor of protein glycosylation (7, 16). However, the increases in \([\text{Ca}^{2+}]_i\) observed in response to tunicamycin occurred more rapidly than can be explained by changes in the glycosylation state.

**Fig. 7.** Effect of extracellular nickel (Ni\(^{2+}\)) on agonist responses in presence of extracellular \(\text{Ca}^{2+}\). Cells were treated with 1 \(\mu\text{M}\) bradykinin (BK; A), 1 \(\mu\text{M}\) TG (B), or 10 \(\mu\text{g/ml}\) TM (C) in \(\text{Ca}^{2+}\)-complete HBBSS in presence and absence of 5 mM NiCl\(_2\). At indicated times, agonists were rapidly rinsed out by rapid buffer exchange with \(\text{Ca}^{2+}\)-complete HBBSS with or without 5 mM NiCl\(_2\); A: \(\text{Ca}^{2+}\)-complete HBBSS containing 5 mM NiCl\(_2\) was rapidly replaced with \(\text{Ca}^{2+}\)-complete HBBSS at end of experiment. Data are given as a representative experiment for A and B and as means ± SE (n = 3 experiments) for C. Con, control.
of proteins. Tunicamycin also inhibits protein palmitoylation (16, 17). This can occur with a time course of several minutes, depending on the half-life of this dynamic posttranslational process (16, 17). We recently found that tunicamycin inhibited palmitoylation of the endothelial isoform of nitric oxide synthase in this model (4). It is possible that proteins involved in capacitative Ca\(^{2+}\) influx are regulated by palmitoylation and are sensitive to tunicamycin. Future studies will be required to determine whether the mechanism by which tunicamycin stimulated influx involved inhibition of protein palmitoylation. Tunicamycin also stimulated nitric oxide synthesis in this model (4). However, nitric oxide probably had no influence on Ca\(^{2+}\) influx by which tunicamycin stimulated influx involved inhibition and are sensitive to tunicamycin. Future studies will be required to determine whether the mechanism by which tunicamycin stimulated influx involved inhibition of protein palmitoylation. Tunicamycin also stimulated nitric oxide synthesis in this model (4). However, nitric oxide probably had no influence on Ca\(^{2+}\) influx in the present study, since the tunicamycin-stimulated increase in [Ca\(^{2+}\)] was not diminished when N\(^{6}\)-monomethyl-L-arginine was used to inhibit nitric oxide synthesis (data not shown).

The increase in [Ca\(^{2+}\)] stimulated by tunicamycin was characterized by a significant lag period in the presence of extracellular Ca\(^{2+}\). This delay ranged from seconds to minutes, depending on the concentration of tunicamycin (4). Although the mechanism responsible for this lag is not known, it might involve time-dependent metabolism of tunicamycin to a more active species or time-dependent alteration of metabolic processes such as protein palmitoylation. Alternatively, partitioning of tunicamycin in the membrane may have required seconds to minutes before threshold concentrations were reached. Importantly, the response to tunicamycin was very rapid when extracellular Ca\(^{2+}\) was absent. This finding indicates that intracellular stores of Ca\(^{2+}\) were accessible to tunicamycin and immediately mobilized in the absence of extracellular Ca\(^{2+}\). It also suggests that the membrane association of tunicamycin might be dependent on the presence of Ca\(^{2+}\) or other divalent cations. In fact, we observed a greater delay in the tunicamycin response in the presence of extracellular nickel than in its absence.

We conclude from these studies that tunicamycin increases the permeability of a discrete intracellular pool of Ca\(^{2+}\), leading to depletion of this store and activation of Ca\(^{2+}\) influx across the plasma membrane. Although the identity of this pool is not known, we have shown that it is depleted by thapsigargin and does not require inositol trisphosphate for mobilization. Several recent studies demonstrate the presence of an intracellular store of Ca\(^{2+}\) that is insensitive to inositol trisphosphate, sensitive to thapsigargin, and regulated by the ryanodine receptor in vascular endothelium (5, 11, 23, 24). In addition, the presence of immunoreactive protein for the ryanodine receptor and its mRNA has recently been confirmed by Western and Northern blots in bovine aortic endothelial cells (14). The endogenous ligand for the ryanodine receptor in mammalian cells is reported to be cyclic ADP-ribose (1, 13, 15, 20). Additional studies will be required to determine whether tunicamycin, which bears some structural resemblance to cyclic ADP-ribose (7, 13), increases [Ca\(^{2+}\)] by a similar mechanism.

Regardless of the precise mechanism by which tunicamycin increased [Ca\(^{2+}\)], these novel and unexpected effects of tunicamycin have several important ramifications. First, alterations in [Ca\(^{2+}\)] may be responsible, in part, for the toxicity of tunicamycin and related species in animal models. Second, these findings suggest that the effects of tunicamycin on glycosylation and palmitoylation may be related to its effects on Ca\(^{2+}\) metabolism. Finally, studies in which tunicamycin has been used as an inhibitor of glycosylation and palmitoylation should be reevaluated in light of this agent’s potential to alter Ca\(^{2+}\) homeostasis.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-51183 and HL-54149.

Address for reprint requests: B. J. Buckley, Dept. of Pharmacology, Box 3813, Duke University Medical Center, Durham, NC 27710.

Received 21 March 1997; accepted in final form 26 June 1997.

REFERENCES


