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Monovalent Ionophores Inhibit Acetylcholinesterase Release from Cultured Chick Embryo Skeletal Muscle Cells

HENRY SMILOWITZ

Department of Pharmacology, University of Connecticut Health Center, Farmington, Connecticut 06032

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SUMMARY

SMILOWITZ, HENRY: Monovalent ionophores inhibit acetylcholinesterase release from cultured chick embryo skeletal muscle cells. *Mol. Pharmacol.* **16**, 202-214 (1979).

Acetylcholinesterase (ACHE) is a glycoprotein that is released in large amounts from embryonic chick muscle *in vivo* and *in vitro*. We show in this paper that the ionophores that transport monovalent cations and divalent cations markedly affect the accumulation and release of ACHE. ACHE release can be stimulated by low levels of the calcium ionophore A23187 and inhibited by low levels of the carboxylic ionophores which transport monovalent cations. Release of ACHE is inhibited 50% by 0.1 μM Monensin, or 0.05 μM Nigericin and is stimulated maximally by 0.05–0.1 μM A23187. The inhibition of ACHE release by the ionophores and the accompanying accumulation of ACHE activity in the cell is rapid and reversible; the inhibition of ACHE release by the ionophores is not due to an overall inhibition of protein synthesis.

The ionophores Monensin and Nigericin cause large membranous vesicles to appear in the vicinity of the muscle nucleii. These appear at ionophore concentrations that are 50 to 500 times lower than those previously reported to swell the Golgi of smooth muscle and immunoglobulin secreting cells. The possibility is raised that the membranous vesicles we observe after ionophore treatment are also derived from the golgi of skeletal muscle and represent sites of intracellular ACHE accumulation and transport.

INTRODUCTION

Chick embryo pectoral muscle cells fuse to form spontaneously contracting multinucleated myotubes in cell culture. These myotubes possess many of the properties of embryonic muscle (for review see Hauschka, 1972 [1]). For example, unlike adult myotubes, the cultured cells possess a high density of acetylcholine receptors (ACHR)[1] all over their surface (2, 3), they exhibit diffuse ACHE staining throughout the sarcolemma (4, 5), and they release large amounts of ACHE into the culture medium (4).

This paper focuses on the release of enzymatically active ACHE from avian myotubes in cell culture. Very little is known about this process. It has been shown to be a continual process (4) and after 24 hours, the amount of ACHE that is released into the cell culture medium varies from 1.3–2.4

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[1] The abbreviations used are ACHE, acetylcholinesterase ACHR, acetylcholine receptors; isoOMPA, tetraisopropyl pyrophosphoramide BCHE, pseudocholinesterase; DFP, diisopropylfluorophosphate; MEM, Eagle's Minimal Essential Medium; TCA, trichloroacetic acid; HEPES *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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times the amount that is found in the cells (4); most of the enzyme that is synthesized is released. The release process requires protein synthesis and is fully inhibited by cycloheximide within 2–3 hours after the complete inhibition of protein synthesis (6). The release process is subject to regulation; electrical stimulation of cultured myotubes results in a decrease in the rate of ACHE release (7).

ACHE has been found in the cisternae of the endoplasmic reticulum of adrenal chromaffin cells (8) and cultured embryo muscle cells (9) by histochemical staining techniques. Hence, it is possible that ACHE is transported to the cell surface in membranous vesicles and released in a manner similar to collagen release. Pro-collagen is assembled within the Golgi, transported within secretory granules and released from the cell by exocytosis (10, 11). Alternatively, it is possible that ACHE is transported to the cell surface in membranous vesicles and then sloughed off the exterior of the cell by proteolysis (12). There is no compelling evidence to suggest that either release mechanism proposed is correct.

We show in this paper that ACHE release is reversibly inhibited by the carboxylic ionophores that transport monovalent cations (Monensin, Nigericin) (13). Previously, the monovalent ionophores have been shown to inhibit plasma cell immunoglobulin secretion (14). The effect of the ionophores is not due to an inhibition of protein synthesis. Rather, the ionophores exert a reversible inhibition of ACHE release which results in the cellular accumulation of enzyme and the appearance of distended membranous vesicles in the vicinity of the nuclei of the myotube. The monovalent ionophores have been shown to alter the golgi apparatus of several cell types, including smooth muscle cells (15), immunoglobulin secreting plasma cells (14) and the chief cells of the rat parathyroid gland (16). Further studies are needed to determine if the expanded membrane vesicles seen in our cells are derived from the golgi. We raise the exciting possibility that in the presence of the monovalent ionophores, ACHE becomes trapped in a discrete step in its intracellular transport.

A preliminary communication of this work has already appeared (17).

MATERIALS AND METHODS

Preparation of the cultures has been described by Fischbach (18). The plating medium (100 ml of 5% MEM) was composed of Eagle's Minimal Essential Medium (80% by volume), heat inactivated horse serum (10% by volume), a saline chick embryo extract (5% by volume), glutamine (2 μM), penicillin (50 units), streptomycin (50 μg) and 1 ml MEM 100 X vitamin solution. The cells were transferred, after two to three days, to an identical medium except for the following changes: the concentration of embryo extract was reduced to 2% (2% MEM) and cytosine arabinoside (50 μM) was added. After two days the cells were maintained in 2% MEM without cytosine arabinoside. Total protein varied less than 10% between dishes plated at the same time. Eagle's MEM, glutamine, penicillin, streptomycin and 100 X vitamin solution were purchased from GIBCO. Horse serum was purchased from Microbiological Associates and saline chick embryo extract was prepared according to Fischbach (18).

Acetylcholinesterase activity was measured spectrophotometrically by the technique of Ellman (19) as modified by Fluck and Strohman (5) and Wilson *et al.* (4). Our assays were routinely done in the presence of 0.1 mM isoOMPA to inhibit BCHE. Pseudocholinesterase activity, as measured by the hydrolysis of butyrylthiocholine iodide, only accounts for 5% of the total enzyme activity in our cultures. The inhibitor, BW284c51 (obtained from Burroughs Wellcome and Co.) when added at 10 μM, inhibits 90% of the acetylthiocholine iodide hydrolyzed.

Medium was pre-treated with DFP as described by Wilson *et al.* (4) to reduce endogenous ACHE and BCHE activity to approximately 2–3% of the untreated media. After 24–48 hr the added DFP had been almost completely hydrolyzed and would not reduce the activity of added ACHE in control experiments. Aliquots of

the DFP treated culture media were removed from the cell cultures and used to assay released ACHE.

The muscle cell cultures were washed with MEM balanced salt solution, scraped in a small volume of 1 M NaCl, 10 mM Tris, 0.5% Triton and 0.2 mM EDTA pH 7.8 (20) and homogenized in a dounce homogenizer. Aliquots were assayed for ACHE activity. This enzyme activity in the cell layer reflects cellular ACHE plus any ACHE bound to the extracellular matrix (9).

Total cell protein was measured by the method of Lowry (21). Total cellular protein synthesis was measured by the incorporation of [^{3}H]leucine or S^{35} Methionine into cold TCA insoluble material. Ten μCi of [^{3}H]leucine (81.8 Ci/mMol) was added to MEM-minus leucine medium supplemented with 3% horse serum and 2% chick embryo extract (22). Incorporation was followed over a four hour period and culture dishes were briefly washed with HEPES buffer pH 7.4, the cells were scraped directly in cold 10% TCA and filtered on 0.45 μ Millipore filters (HAWP). Similarly, 50 μCi of S^{35} Methionine (500 Ci/mMol) was added to 2% medium. Incorporation was followed over a 24 hour period of time, after which the cells were treated as above.

Electron microscopy was performed by the University of Connecticut Health Center Central Electron Microscopy Facility. Week old chick embryo muscle cell cultures were glutaraldehyde fixed (2% for 30 min), followed by osmium post fixation, ethanol dehydration and embedding in epon. Suitable areas of the specimen were cut out, remounted on epon blocks, sectioned *en face* and stained with uranyl acetate and lead citrate. Magnification is 27,000.

The ionophores were obtained as follows: Monensin M.W. 670 (Eli Lilly Co.), Nigericin M.W. 740 (Eli Lilly Co.), X537A M.W. 590 (Hoffman-LaRoche, Inc.) and A23187 M.W. 523 (Eli Lilly Co.). They were dissolved in 100% ETOH at 1 mg/ml or 1 mM and stored at −20°. They were added to the culture media as needed—but never in amounts that exceeded 1γ ETOH per ml of media. At this level, ETOH had no measurable effect on ACHE release or total cell protein synthesis.

RESULTS

The amount of acetylcholinesterase (ACHE) released from avian skeletal muscle cells grown in cell culture as a function of time is shown in Figure 1A. The release of ACHE is linear up to 80 hours. Figure 1B shows the total amount of ACHE activity that was released into the culture medium (per 24 hours) of one week old muscle cell cultures when seeded at densities ranging from 0.5×10^5 to 5.0×10^5 cells per dish. The release of ACHE is proportional to cell density in this range. In the experiments which follow, we have used one week old muscle cell cultures plated at cell densities ranging from 2×10^5 to 5×10^5 cells per 35 mM dish. We have studied the effects of the ionophores Monensin, Nigericin and A23187 on the amount of ACHE activity that is released and the amount of ACHE activity that is found in the cell layer.

Monensin. Figure 2 shows the effect of varying concentrations of Monensin on the release of ACHE and the corresponding cellular levels of the enzyme. The values in Figure 2 are expressed as a percent of the value seen in muscle cell cultures that contain no drug. The concentration of Monensin which inhibited ACHE release 50% was 0.13 μM. At 0.1 μM there was a maximal (28%) increase in cellular ACHE activity.

Nigericin. Figure 3 shows the effect of varying concentrations of Nigericin on the release of ACHE and the corresponding cellular levels. The concentration of Nigericin which inhibited release 50% was 50 nM. At 40 nM, there was a maximal (34%) increase in cellular ACHE activity. Figure 4 shows a similar experiment in which both low and very high concentrations of Nigericin were used and total protein synthesis was measured. In this experiment ACHE release was inhibited 73% by 40 nM Nigericin while cellular accumulation was 18% of control and total protein synthesis was 90% of control values. However at higher Nigericin concentrations (1 μM) maximal inhibition of ACHE release was 84% while cellular levels remained nearly normal and total protein synthesis was inhibited 80%.

A23187. Figure 5 shows the effect of varying concentrations of A23187 on the release of ACHE activity and the corresponding

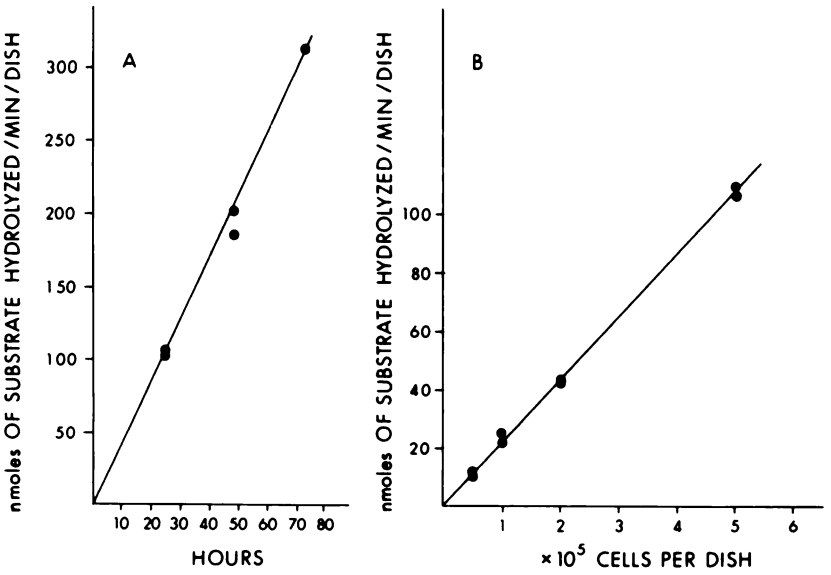

FIG. 1. *Release of ACHE as a function of time and of cell density*

Panel A) Week-old myotubes were incubated in DFP treated medium. Aliquots were assayed for ACHE activity at 24, 48 and 72 hr. The ordinate represents total enzyme activity released per dish.

Panel B) Release of ACHE as a function of plating cell density. Muscle cell cultures were plated at densities ranging from 0.5–5.0 × 10^{-5} cells/dish. When the cultures were one week old, the amount of ACHE that was excreted into DFP medium over 24 hr was assayed. The ordinate represents total enzyme activity released per dish. In both A and B, each point represents a single culture dish.

cellular levels. At low concentrations of A23187 there was a marked increase in the release of ACHE activity while cellular ACHE activity was unaffected. At 5 × 10^{-8} to 10^{-7} M A23187, ACHE release was 140% of control values while cellular levels were unchanged. At higher concentrations of A23187, both the release of ACHE and cellular levels of ACHE were markedly reduced. In a separate experiment the cells were incubated with 1 μM A23187. The ACHE activity found in the cell layer was 45.6 ± 3 and 14.0 ± 1 moles of substrate hydrolyzed per min/mg protein (3 determinations) for control and ionophore treated cultures, respectively. Similarly, the ACHE activity released into the culture medium was 53.0 ± 3 and 32.7 ± 5 nMoles of substrate hydrolyzed per min per mg protein (3 determinations) for control and A23187 treated cultures, respectively. Total protein synthesis was inhibited 13.4%, 10.2%, 20% in three separate determinations.

Mechanism of ionophore action: Protein synthesis. The inhibition of ACHE release by the ionophores Monensin and Nigericin did not result from an inhibition of total cell protein synthesis. For example, the overall rate of protein synthesis (determined as described in METHODS) was inhibited only 10% by 40 nM Nigericin (see Fig. 4) while ACHE release was inhibited 73% and ACHE accumulation in the cell layer was 118% of control values. This suggests a preferential effect of Nigericin on ACHE release that did not involve overall protein synthesis. This is further illustrated in Fig. 6 in which the kinetics of the inhibition of ACHE release by Nigericin, Monensin and protein synthesis inhibitors are compared. Figure 6 shows the rate of ACHE release (as measured by ACHE activity in the culture media) at hourly intervals in control cultures and after the addition of: 50 nM Nigericin, 10 μM Monensin, 20 μg/ml Puromycin (20 μg/ml Puromycin inhibits total protein synthesis by 98% within 5 min) (23) or 100 μg/ml Cycloheximide (100 μg/ml Cycloheximide inhibits total protein synthesis by 85% within 10 min) (23). There are three culture dishes in each

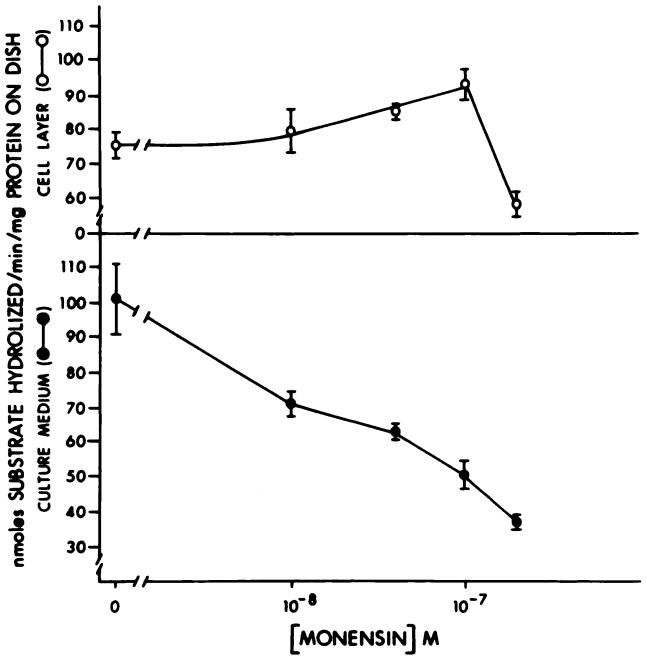

FIG. 2. *Effect of [Monensin] on the release of ACHE and the corresponding cellular level*

●—●— represents the effect of Monensin on the release of ACHE after 18 hours of incubation with Monensin. The upper panel (O—O—) represents the effect of Monensin on cellular level. The ordinate is expressed as nmoles of substrate hydrolyzed per min per mg of protein on the dish at 25°. Each point represents the mean of three experimental determinations. The standard error of the mean is indicated.

group. Zero time represents the amount of ACHE released into the culture medium for one hour prior to the addition of drugs. The one hour time point represents the amount of ACHE released into the culture medium for one hour after the addition of drugs. The second and third hour points each represent the amount of ACHE released in subsequent one hour intervals. During the first hour after the administration of Puromycin or Cycloheximide there was little effect on the rate of ACHE release as compared to control cultures. Nigericin and Monensin treated cultures however exhibited a 50% inhibition of ACHE release during the first hour. The differences between Nigericin, Monensin, Puromycin and Cycloheximide treated cultures largely disappeared between the first and second hour. By the third hour, ACHE release was maximally inhibited by all four drugs.

Mechanism of ionophore action: Reversibility. At 40 nM Nigericin the inhibition of ACHE release was approximately 50%. Table 1 shows that after a 14 hour treatment with 40 nM Nigericin, removal of the drug resulted in an increased release of ACHE (.34 to .82 of the control value) and decreased cellular activity of ACHE (1.3 to 0.84 of control value) within 4 hours. These results indicate that the effect of Nigericin is reversible.

Mechanism of ionophore action: Ultrastructural changes. Very low concentrations of the ionophores Nigericin and Monensin produce specific ultrastructural changes in our cells. Electron microscopy revealed large membranous vesicles in the vicinity of nucleii in ionophore treated cells (Fig. 7) but not in control cells (Fig. 8). Similar effects were seen with both Nigericin (50 nM) and Monensin (0.1 μM). Fifty to five hundred fold higher concentrations of the monovalent ionophores have been observed to produce ultrastructural changes of the Golgi of smooth muscle cells (15) and plasma cells (14). It is possible that the membranous vesicles that we observe represent ultrastructural changes in the Golgi of skeletal myotubes in culture but this

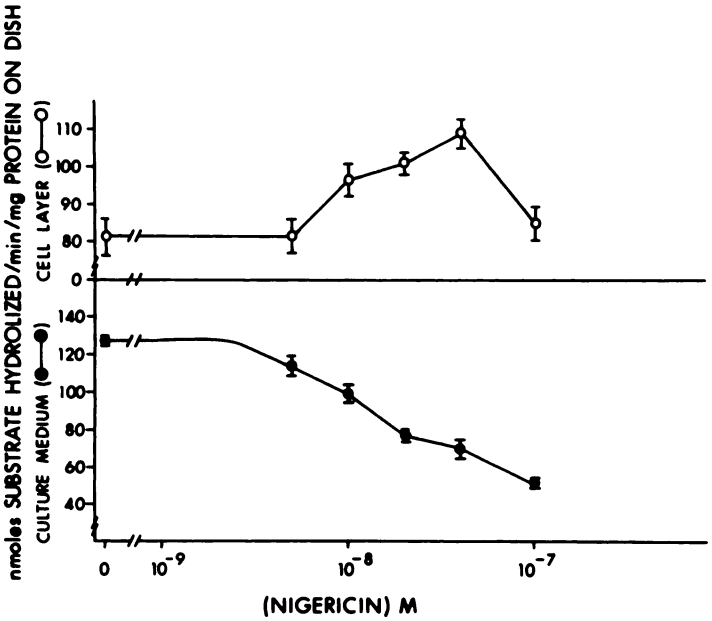

FIG. 3. *Effect of Nigericin on release of ACHE and on cellular levels*

Effect of Nigericin on the release of ACHE (—●—●—) (lower panel) and the corresponding cellular levels (—O—O) (upper panel) after 18 hours of incubation. With Nigericin the ordinate is expressed as nmoles of substrate hydrolyzed per min per mg of protein on the dish at 25°. Each point represents the mean of three experimental determinations. The standard error of the mean is indicated.

must be proved. The inhibition of ACHE release may be related to the observed ultrastructural changes.

DISCUSSION

The carboxylic ionophores Monensin and Nigericin are capable of transporting monovalent ions across cell membranes down their concentration gradients (13). Their ion selectivities are not the same; Monensin is ten times more selective for Na^+ ions than K^+ ions while Nigericin is 45 times more selective for K^+ ions than Na^+ ions. The carboxylic ionophore A23187 is far more selective for divalent cations (13). Our data show that acetylcholinesterase (ACHE) release is inhibited by the ionophores which transport monovalent ions.

The absolute amount of ACHE activity that is present in these cells and released from these cells varies from plating to plating. Similarly, the amount of inhibition of ACHE release by the ionophores and the attendant ACHE accumulation is variable in different sets of culture dishes. For example, Fig. 3 shows that ACHE release was inhibited 50% by 50 nM Nigericin while Fig. 4 shows that ACHE release was inhibited 70% by 40 nM Nigericin. However, the results within a particular set of dishes are very reproducible. Therefore, we have not pooled our data from the many experiments we have done with each drug but instead present representative experiments. The exact quantitative data varies from cell plating to cell plating—but the same qualitative effect is always observed with a particular drug.

The inhibition of ACHE release by the ionophores is not due to an overall inhibition of protein synthesis. For example, direct measurements of protein synthesis by determining the uptake of S^{35} Methionine into TCA insoluble material show that at 40 nM Nigericin protein synthesis is inhibited 10% while ACHE release is inhibited 70% (fig. 4). Furthermore, the inhibition of ACHE release observed after the addition of 50 nM Nigericin is very rapid. There is greater than 50% inhibition within the first hour after Nigericin addition. However, in the presence of inhibitors of protein

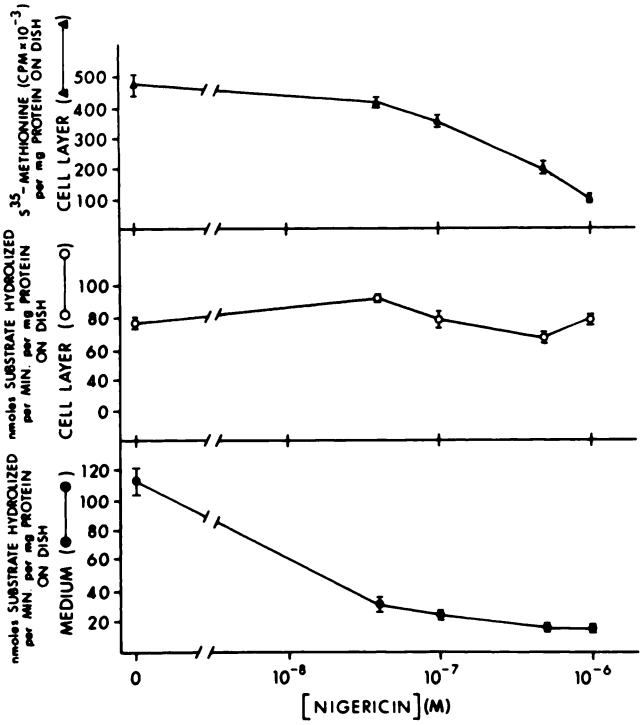

FIG. 4. *Effect of Nigericin on release of ACHE, cellular levels, and S^{35} methionine incorporation*
Release of ACHE (—●—●—), lowest panel, and the corresponding cellular levels (—○—○—) middle panel, and on the corresponding S^{35} Methionine incorporation (—▲—▲—), top panel, after 18 hours of incubation with Nigericin. Each point represents the mean of three experimental determinations. The standard error of the mean is indicated. S^{35} Methionine (3.3 μC/ml) was added to each culture dish at the time of drug addition. Incorporation was linear over the duration of the experiment. The values shown represent the total incorporation into cold TCA insoluble material after 18 hour incubation with Nigericin.

synthesis such as Cycloheximide (23) (100 μg/ml, which inhibits protein synthesis by 85% within 10 min [23]), or Puromycin (20 μg/ml, which inhibits protein synthesis by 98% within 5 min [23]), ACHE release is unaltered for the first hour. This indicates that the effects of the carboxylic ionophores are not due to depletion of cellular ACHE. Rather it appears that enzyme that is normally released is not getting out of the cell in the presence of the monovalent ionophores.

The same type of result has been obtained for Monensin. Direct measurements of protein synthesis in four separate experiments indicated that cultures treated with 0.1 μM Monensin exhibited 100%, 86%, 90% and 88% of the [^{3}H]leucine incorporation of their controls. At 0.1 μM Monensin, ACHE release is inhibited 64% and this inhibition of ACHE release is very rapid. There is a greater than 50% inhibition of ACHE release within the first hour of drug addition.

Under conditions in which ACHE release is inhibited one would expect to get an accumulation of cellular ACHE. In fact such an accumulation is observed. At 40 nM Nigericin, ACHE release is found to be inhibited 45 and 70% (figure 3 and 4) while cellular accumulations are 133% and 118% of control values. Since a certain percent of the ACHE found in the cell layer may be bound to extracellular matrix, the percentage increase observed in the cells may be an underestimate. Nevertheless, there is a discrepancy between the amount of enzyme activity that accumulates in the cell and the amount by which the enzyme activity in the culture medium is decreased. This may reflect a variety of complex phenom-

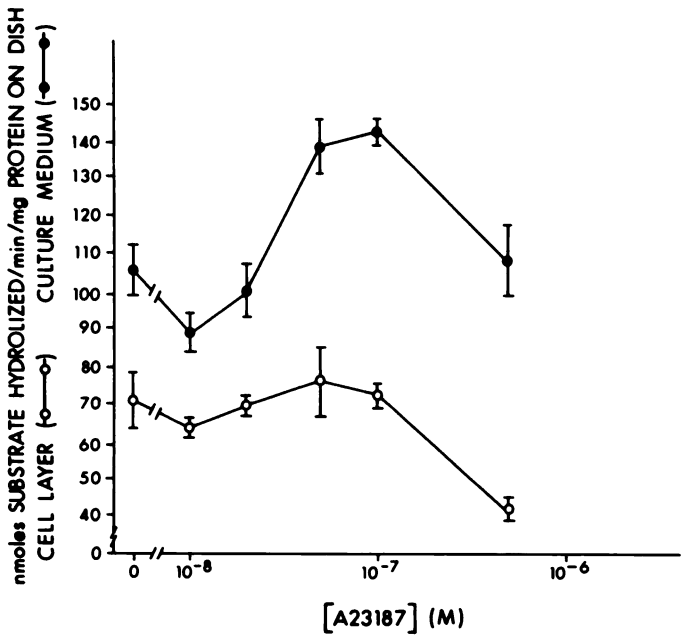

FIG. 5. *Effect of A23187 on release of ACHE and cellular levels*

Release of ACHE (—●—●—) and the corresponding cellular levels (—○—○—) after 18 hours incubation with A23187. Each point represents the mean of three experimental determinations. The standard error of the mean is indicated.

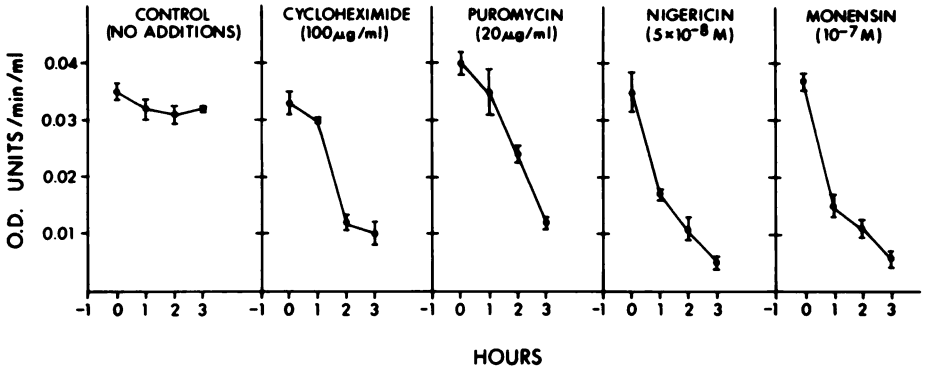

FIG. 6. *Kinetic patterns of inhibition of nigericin, monensin, cycloheximide, and puromycin*

Time course of the inhibition of ACHE release by: 40 nM Nigericin, 10 μM Monensin, 100 μg/ml Cycloheximide and 20 μg/ml Puromycin. The amount of ACHE that is released into the culture medium (expressed as O.D. units/min/ml) in hourly intervals is shown. The interval −1 to 0 represents the one hour interval before any drugs were added. The interval 0 to 1 represents the first hour interval after drug addition, etc. Each point represents the mean of three experimental determinations. The standard error of the mean is indicated.

ena such as enzyme degradation and turnover or feedback inhibition of enzyme synthesis.

While the mechanism by which ACHE release is inhibited and the mechanism by which ACHE accumulates in the cell are not understood, it is clear that both are reversible events. Nigericin 50 nM inhibited ACHE release by approximately 50% while cellular accumulation of ACHE was as high as 130–140% of normal. Yet within 2 hours of ionophore removal, ACHE release and cellular levels were nearly normal.

The monovalent ionophore X537A has

TABLE 1

Reversibility of the Nigericin effect on ACHE release and accumulation

One-week-old myotube cultures were incubated in DFP treated medium for 14 hours. Half the dishes received 40 nM Nigericin. The amount of ACHE released into the medium and the amount found in the cells after 14 hours incubation is reported in A. Each value represents three separate culture dishes. The mean and standard error of the mean are indicated. The drug was removed and new DFP treated medium was added to the remaining dishes. After 2 hours, the medium and cell layer was assayed for ACHE activity (B). New DFP treated medium was added to the remaining dishes. After 2 more hours, the medium and cells were assayed for ACHE (C). The Students *t*-test was used to test the hypothesis that the means of the two groups (control and nigericin) are different.

	Total protein/ dish	nMoles substrate hydrolyzed/min/mg protein on dish			
		Medium	Ratio	Cells	Ratio
	(Mg)		*(Nig/Cont)*		*(Nig/Cont)*
A. 14 hr incubation					
Control	401.67 ± 15.9	8.65 ± 0.72		30.59 ± 1.09	
	(p = >0.2)	(p = <0.02)	0.61	(p = >0.05)	1.15
Nigericin	411 ± 15.31	5.32 ± 0.15		35.163 ± 1.63	
B. *Drug Free* (0–2 Hour)					
Control	401.67 ± 15.9	8.65 ± 0.72		30.59 ± 1.09	
	(p = >0.2)	(p = <0.02)	0.61	(p = >0.05)	1.15
Nigericin	411 ± 15.31	5.32 ± 0.15		35.163 ± 1.63	
C. *Drug Free* (2–4 Hour)					
Control	423.3 ± 11.67	7.20 ± 0.73		30.92 ± 3.33	
	(p = >0.2)	($p > 0.2$)	0.82	($p > 0.2$)	0.84
Nigericin	423.3 ± 19.3	5.88 ± 0.61		25.92 ± 3.27	

been shown to markedly alter the ultrastructure of the Golgi apparatus of smooth muscle cells (15). Monensin and Nigericin cause similar alterations of the Golgi of immunoglobulin secretory plasma cells (14). Although the concentrations of ionophores used were 50 to 500 times greater than the concentration required to inhibit ACHE release maximally in this study, it is possible that the ultrastructural changes that have been observed with X537A, Monensin and Nigericin also occur in skeletal muscle and contribute to the inhibition of ACHE release that we observed. Our data indicate that Monensin and Nigericin both lead to a rapid buildup of membranous vesicles inside our cultured skeletal muscle cells (Figs. 7 and 8). Our recent studies (unpublished) show that these vesicles are evident by two hours after drug treatment. Since the Golgi are fairly inconspicuous membranes of the sarcoplasm of normal muscle, it is difficult to know whether the membrane vesicles we see are derived from Golgi. It will be necessary to use histochemical stains for enzyme markers of the Golgi to answer this question. It will be very interesting to determine if the ACHE that accumulates in ionophore treated cells is largely localized in these vesicles. If so, these membranes may represent a discrete step in the intracellular transport pathway of ACHE, which can be ultimately isolated and characterized.

The effects of the divalent ionophore A23187 are markedly different from those of the monovalent ionophores. At low concentrations of A23187, ACHE release is stimulated while cell levels remain normal. At higher concentrations both ACHE release and cellular levels decrease. It is possible that low levels of the calcium ionophore stimulate ACHE release by stimulating the exocytosis process (13). There is ample evidence to show this occurs in secretion systems in which A23187 stimulates membrane fusion and exocytosis of membrane vesicles (13, 24). If true, this would support the view that ACHE release utilizes a membrane fusion-exocytotic process, akin to collagen and immunoglobulin release, rather than a protease-stimulated slough-

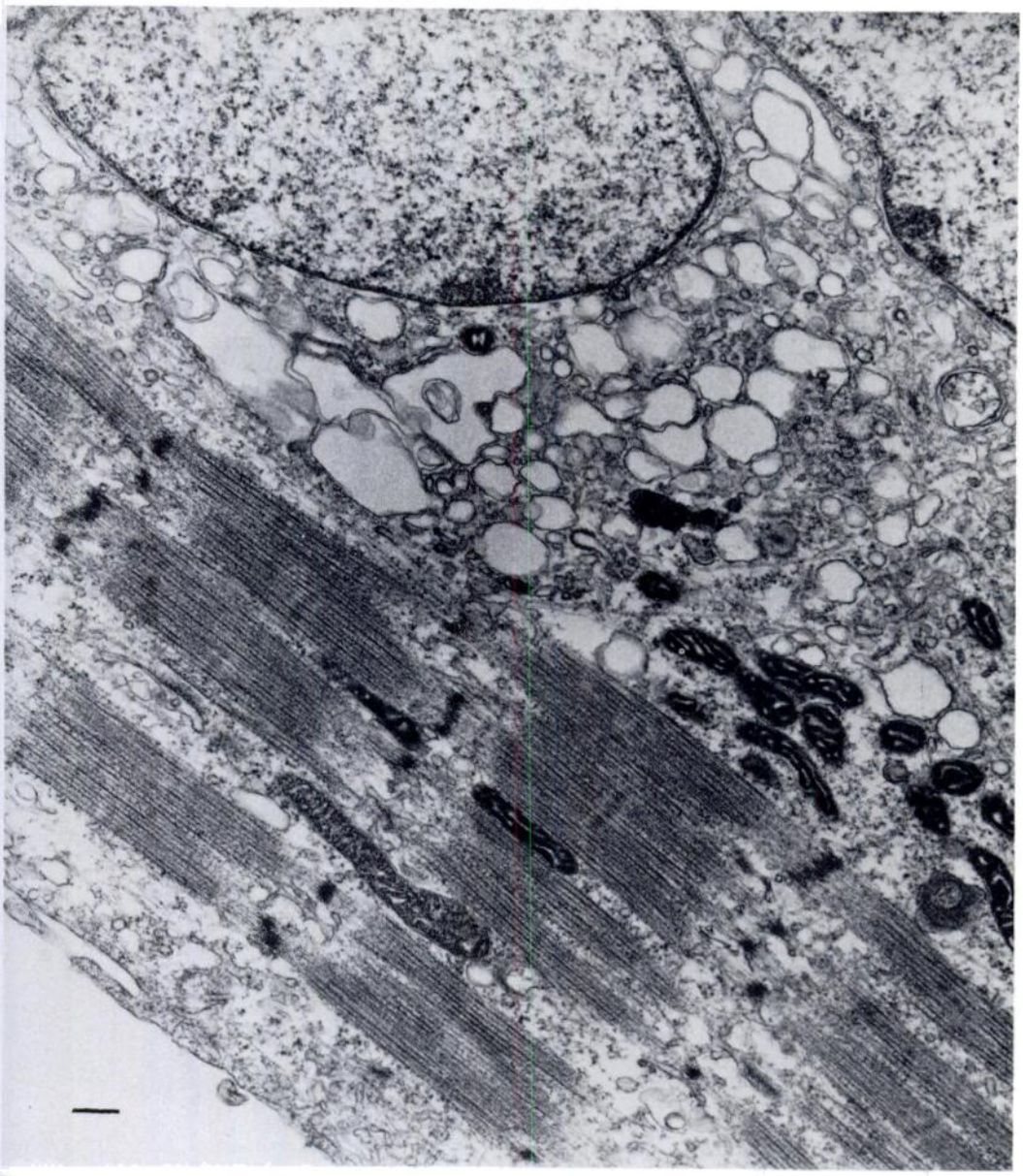

FIG. 7. *Ultrastructural changes in cells treated with Nigericin*

Week-old chick embryo muscle cultures were treated with 50 nM Nigericin overnight. Cultures were fixed with glutaraldehyde, followed by osmium, ethanol dehydration and embedding in epon. Suitable areas of the specimen were cut out, remounted on epon blocks, sectioned *en face* and stained with uranyl acetate and lead citrate. Magnification is 26,125. Performed by University of Connecticut Health Center Central Electron Microscopy Facility. 0.675 CM represents 250 nM.

ing phenomenon. The data also suggest a stimulation of ACHE synthesis by low levels of A23187, perhaps reflecting a complex feedback system in which enzyme release and synthesis are linked.

High levels of calcium ionophore, however, may selectively inhibit the synthesis of ACHE as well as other membrane bound proteins. We offer this as a possibility since overall cell protein synthesis was only in-

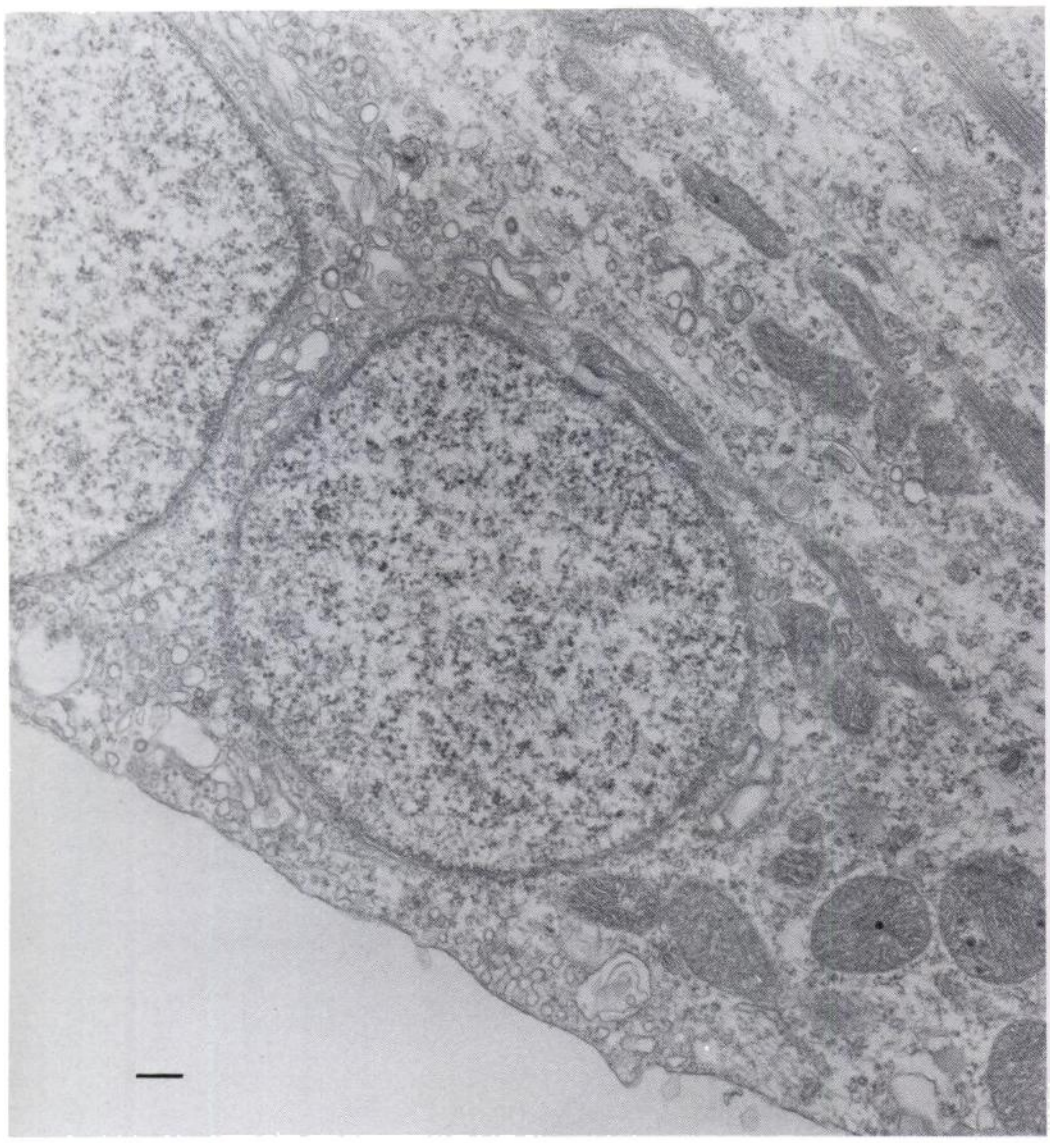

FIG. 8. *Absence of pathologic changes in untreated cells*

Control cultures. Magnification is 25,300. Performed by the University of Connecticut Health Center Electron Microscopy Facility. 0.675 CM represents 250 nM.

hibited 13.4%, 10.2% and 20% by 1 μM A23187 (in three separate determinations) while ACHE release and cell levels were inhibited 38% and 70% respectively in one set of experiments (see RESULTS) and 40% and 60% in another set of experiments (data not presented). Another protein whose synthesis appears to be very sensitive to micromolar concentrations of calcium ionophore is Ca^{++} ATPase (25). If in fact micromolar levels of Ca^{++} ionophore selectively block the synthesis of ACHE, some of the developmental aspects of ACHE may be explained. For instance, it has been shown that embryonic, denervated and dystrophic muscle all possess high rates of ACHE synthesis and release while adult or electrically stimulated muscle possess low levels of ACHE synthesis and release (26). Our experiments with the calcium ionophores

A23187 raise the possibility that ACHE synthesis can be regulated by intracellular free calcium levels. We speculate that ACHE synthesis is normally regulated by intracellular free Ca^{++} levels which is, in turn regulated by muscle activity (27). Thus the low level of ACHE synthesis in adult muscle may be due to high intracellular calcium levels in active muscle. The concentration of ACHE at the muscle endplate, then, may reflect a special site where ACHE release is decreased or where ACHE is trapped by specialized structures such as basement membrane (28).

The monovalent ionophores inhibit the release of other glycoproteins that are continually released from cells. Immunoglobulin release (14), for example, is sensitive to the monovalent ionophores. Collagen and fibronectin release from human fibroblasts is sensitive to the monovalent ionophores at 0.1 μM concentrations (29). The inhibition of collagen release by 1 μM Monensin has also been demonstrated (30). The fact that the monovalent ionophores inhibit the release of many different secretory proteins from many different cells suggests that this is a general phenomena. It is unlikely that the observed effects of monovalent ionophores are due to specific inhibition of the synthesis of ACHE, collagen, fibronectin and immunoglobulin. At very low concentrations of the monovalent ionophores, total protein synthesis is minimally inhibited while secretion is severely curtailed (Figure 4 and ref. 29). Since total protein synthesis is very sensitive to the energy metabolism of the cell, we find it is difficult to explain the inhibition of ACHE release and collagen and fibronectin release (29) by an overall inhibition of the energy metabolism of the cell (30). We are currently studying the mechanisms by which very low concentrations of the monovalent ionophores can inhibit the secretion of many different glycoproteins.

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