verse the direction of the induced current. Taken together, these results indicate that cyclic AMP injections induce a reversible increase in the membrane sodium current of these neurons.

Arsenazo III was further used to examine possible changes in the voltage-dependent calcium conductance or in cellular calcium regulation during cyclic AMP injections. Figure 2C (left) shows the characteristic increase and subsequent recovery of the signal at 660 nm during and after a voltage step to +20 mV for 1 second. During the following several minutes a total of five intracellular iontophoretic injections of cyclic AMP were administered under voltage clamp, of which three are represented in Fig. 2C (middle). When the voltage was unclamped after an injection, the cell exhibited the nucleotide-induced depolarization that usually elicited action potentials. Immediately after the fifth injection (not shown), the voltage was stepped to +20 mV in the same fashion as before. The absorbance, recorded in Fig. 2C (right), showed no observable change in either the characteristics of calcium influx or its subsequent regulation as reflected by the recovery of the dye signal.

In summary, we were unable to detect changes in resting Ca^{2+} levels of voltage clamped neurons after intracellular cyclic AMP elevation. In addition, no changes were found in the characteristics of calcium influx or its subsequent regulation during and after nucleotide injections. Although cells were studied from each of the six ring ganglia, only the large, identifiable neurons were examined. We have not ruled out the possibility that other neurons in *Archidoris* may exhibit direct cyclic AMP–induced effects on cellular calcium parameters, like those in certain *Aplysia* neurons (*6*). While the absence of cyclic nucleotide–induced calcium changes in the neurons used in this study indicates that there are no direct effects, it should be stressed that increased intracellular cyclic AMP levels will have a large indirect effect on internal $[Ca^{2+}]$ under physiological conditions. That is, the steady inward current induced by cyclic AMP will depolarize the membrane of unclamped neurons and thereby generate action potentials with corresponding calcium influx.

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Independent Pathways for Secretion of Cholesterol and Apolipoprotein E by Macrophages

Abstract. *Cholesterol-loaded macrophages secrete cholesterol and apolipoprotein E. The current studies show that this secretion occurs by two independent pathways. In the absence of serum, the cells secrete apolipoprotein E, but not cholesterol. In the presence of monensin (an inhibitor of protein secretion), the cells secrete cholesterol, but little apolipoprotein E. After secretion, apolipoprotein E and cholesterol associate with high-density lipoprotein to form a particle that can deliver cholesterol to the liver by receptor-mediated endocytosis. We conclude that apolipoprotein E does not function to remove cholesterol from macrophages but rather to participate in "reverse cholesterol transport."*

In humans or animals with hypercholesterolemia, cholesteryl esters accumulate as cytoplasmic lipid droplets in macrophages (*1*). Possible mechanisms for such accumulation have been disclosed by studies of macrophages in vitro. These studies have shown that mouse and human macrophages have surface receptors that allow them to take up and degrade large amounts of plasma lipoproteins that have been chemically altered. For example, when the low-density lipoprotein (LDL) of human plasma is modified by acetylation or treatment with malondialdehyde, the modified lipoprotein binds to receptors on cultured macrophages and is taken up by endocytosis and delivered to lysosomes (*2*). Within the lysosome the cholesteryl esters of acetyl-LDL are hydrolyzed, and the liberated cholesterol is transferred to the cytoplasm where it is reesterified and stored in cholesteryl ester droplets (*3*).

Under appropriate conditions, macrophages can rapidly secrete the large amounts of cholesterol that they have stored in the cytoplasm. Secretion requires the presence in the culture medium of an agent, such as high-density lipoprotein (HDL), that is capable of binding cholesterol (*4*). In the absence of a cholesterol-binding substance, the cytoplasmic cholesteryl esters are continuously hydrolyzed and reesterified in a sequence termed the cholesteryl ester cycle (*4*). When HDL is present, the hydrolysis of the stored cholesteryl esters continues, but reesterification no longer occurs and the liberated cholesterol is secreted from the cell. Macrophages can secrete huge amounts of cholesterol in this fashion—up to 200 μg per milligram of cell protein per day (*4*).

At the same time that macrophages secrete cholesterol, they also synthesize and secrete large amounts of apolipoprotein E (apo E), a normal protein component of plasma lipoproteins (*5*). First detected in mouse macrophages, the secretion of apo E has also been shown to take place in macrophages derived from circulating human monocytes (*5*). The

apo E emerges from macrophages in association with phospholipid in the form of bilayer discs (*5*). In mouse macrophages the secretion of apo E is stimulated up to tenfold when the cells have been induced to accumulate cholesteryl ester as a result of incubation with acetyl-LDL (*5*).

The simultaneous secretion of cholesterol and apo E raised the question of whether apo E plays a direct role in mediating cholesterol secretion, or whether it serves some other purpose. The current studies were designed to explore this question through the use of a quantitative immunoprecipitation assay for ^{35}S-labeled apo E secreted by macrophages.

A rabbit was immunized with apo E that had been isolated by preparative sodium dodecyl sulfate (SDS)-gel electrophoresis from delipidated mouse very low density lipoproteins (VLDL) (*6*). This antibody gave a single line of identity when tested on Ouchterlony immunodiffusion plates against mouse VLDL, mouse HDL, and purified mouse apo E. Moreover, when mouse serum was subjected to electrophoresis, transferred to nitrocellulose paper, and incubated with the antibody [immunoblotting technique (*7*)], the antibody stained only a 35,000-dalton protein consistent with apo E (*8*). There was no staining of proteins corresponding to the other plasma apoproteins (A-I, A-II, B, or C).

The antibody was used to quantify the amount of [^{35}S]methionine-labeled apo E secreted by macrophages. For this purpose mouse macrophages were loaded with cholesteryl esters by incubation for 24 hours with acetyl-LDL. They were then incubated for a further 24 hours in the absence of lipoproteins so that the cellular cholesteryl ester cycle would reach equilibrium. The cells were then incubated for a further 24 hours in the presence of [^{35}S]methionine. Portions of the medium were incubated with increasing amounts of anti–apo E and then with *Staphylococcus aureus* coated with Protein A (Pansorbin). The immunoprecipitated proteins were eluted from the Pansorbin, and a portion was subjected to scintillation counting. The remainder was subjected to SDS-polyacrylamide gel electrophoresis and then examined by autoradiography. Figure 1 shows that increasing amounts of anti–apo E precipitated a maximum of about 24 percent of the total ^{35}S-labeled proteins secreted by the cholesterol-loaded macrophages. This amount was more than 25-fold greater than the amount precipitated by nonimmune serum. Electrophoresis of the immunoprecipitate showed a single band at a molecular weight of 35,000 consistent with apo E (Fig. 1 inset, lane B).

In a total of 23 experiments, we found that immunoprecipitable ^{35}S-labeled apo E averaged 12 percent of the total protein secreted by cholesteryl ester–loaded macrophages. When the cholesterol loading step was omitted (six experiments), immunoprecipitable ^{35}S–apo E constituted an average of 0.7 percent of the total protein secreted.

Table 1. Dissociation of secretion of cholesterol and apo E in mouse macrophages. Monolayers of unstimulated macrophages were prepared as described in Fig. 1. On day 1, cells received 1 ml of medium A, containing [^{35}S]methionine (50 μCi/ml), fetal calf serum (FCS) (10 percent), and acetyl-LDL (30 μg/ml). On day 2 (zero time), all monolayers were washed. Three dishes were combined and processed for measurement of cholesterol content and [^{35}S]methionine incorporation into cellular protein (group 1). Other sets of three monolayers received 1 ml of medium A containing [^{35}S]methionine (50 μCi/ml) in the absence of FCS (group 2), in the presence of 10 percent FCS (group 3), or in the presence of 10 percent FCS plus 1 μ*M* monensin (group 4). After incubation for 24 hours, on day 3 the media from three replicate dishes were combined, dialyzed against 25 m*M* ammonium bicarbonate (*5*), and analyzed for ^{35}S-labeled apo E by immunoprecipitation as described in Fig. 1. The specific radioactivity of cellular protein was determined by dividing the amount of radioactivity in total cell protein by the mass of total cell protein (*5*). The amount of ^{35}S-labeled apo E secreted into the medium was calculated by dividing the immunoprecipitable radioactivity in the medium by the specific radioactivity of the total cellular protein. The amount of total cholesterol lost from the cells (free plus esterified) was determined by subtracting the value in group 2, 3, or 4 from that in group 1. The cellular content of free and esterified cholesterol was measured by gas-liquid chromatography (*3*, *4*).

Group	Incubation	Cellular cholesterol content (μg sterol/mg protein)		Total cholesterol secreted from cell (μg sterol/mg protein)	^{35}S-Labeled apo E secreted from cell (μg/mg protein)	[^{35}S]Methionine incorporated into cellular protein (10^{-7} × dpm/mg protein)
		Free	Esterified			
1	Zero time	63	196			4.0
	After 24 hours	71	203	0	189	5.4
2	Without serum					
3	With serum	55	64	140	163	5.3
4	With serum and 1 μ*M* monensin	60	99	100	15	4.1

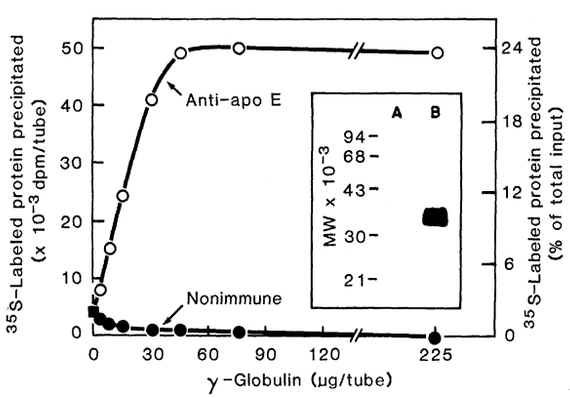

Fig. 1. Immunoprecipitation of ^{35}S–apo E secreted by cholesterol-loaded mouse macrophages. Macrophages were isolated from the peritoneal cavity of unstimulated mice and placed in culture on day 0 as described (*3*, *4*). On day 1, each monolayer (6×10^6 adherent cells per 35-mm dish) received 1 ml of Dulbecco's modified Eagle medium (DMEM) containing acetyl-LDL (100 μg/ml). On day 2, cells were washed and switched to DMEM containing human albumin (1 mg/ml). On day 3, each monolayer received 0.6 ml of medium A (methionine-free DMEM supplemented with 40 μ*M* unlabeled methionine) containing [^{35}S]methionine (200 μCi/ml). On day 4, the media from 12 dishes were combined and dialyzed against 25 m*M* ammonium bicarbonate (*5*). Portions (2×10^5 dpm) of the dialyzed media were incubated in 300 μl of solution containing 20 m*M* tris-Cl (*p*H 7.5), 0.5 percent Triton X-100, 0.25 percent sodium deoxycholate, 0.3 percent sodium dodecyl sulfate (SDS), 0.3*M* NaCl, 0.2 m*M* phenylmethylsulfonyl fluoride, and the indicated amount of one of the following: ■, none; ●, nonimmune rabbit serum (γ-globulin fraction); and ○, rabbit antiserum to apo E (γ-globulin fraction) (*6*). After incubation at 37°C for 2 hours, each tube received 30 μl of a 10 percent (weight to volume) suspension of Pansorbin (Calbiochem) and incubations were continued at 24°C for 15 minutes. After centrifugation, the pellet was washed three times with buffer B (10 m*M* sodium phosphate, *p*H 7.4, 0.5 percent Zwittergent 3-14, 0.5*M* NaCl, 0.1 percent SDS, 10 m*M* dithiothreitol, 2 m*M* methionine, and 5 mg of bovine albumin per milliliter) and once with buffer C (10 m*M* sodium phosphate, *p*H 7.0, and 0.5 percent Triton X-100). The immunoprecipitates were resuspended in 200 μl of buffer O (*5*), heated at 90°C for 3 minutes, and centrifuged. Portions of the supernatants were processed for scintillation counting (*5*) or subjected to electrophoresis on 10 percent SDS-polyacrylamide gels and then fluorography for 72 hours (inset); (lane A) nonimmune serum; (lane B) immune serum. Molecular weight (*MW*) standards are indicated.

To determine whether the secretion of apo E was coupled to the secretion of cholesterol, we performed two maneuvers to dissociate these two events. First, we incubated cholesterol-loaded macrophages in the absence and presence of fetal calf serum. In previous studies, we showed that macrophages do not secrete large amounts of cholesterol in the absence of serum, which contains HDL and other cholesterol-binding molecules (*4*). Second, we incubated the cholesterol-loaded cells in the presence of fetal calf serum plus the carboxylic ionophore monensin. This drug is known to block the movement of secretory proteins from the Golgi apparatus to the plasma membrane (*9*) and hence it would be expected to block the secretion of apo E. To estimate the absolute amount of apo E secreted, we included [^{35}S]methionine in the medium for 24 hours prior to the addition of serum and monensin. During this time the specific radioactivity of cellular proteins reached a steady-state value. During the secretory period, [^{35}S]methionine was maintained in the culture medium at the same specific activity. To calculate the mass of immunoprecipitable ^{35}S–apo E, we assumed that the specific activity of the [^{35}S]methionine in apo E was the same as the steady-state specific activity of [^{35}S]methionine in total cell proteins. To estimate the mass of cholesterol secreted, we measured the decline in cell cholesterol content by gas-liquid chromatography (*3*, *4*).

Table 1 shows that serum and monensin produced a dissociation between the secretion of apo E and of cholesterol. In the presence of serum, the cells secreted almost equal amounts of cholesterol (140 μg per milligram of protein) and ^{35}S–apo E (163 μg per milligram of protein). In the absence of serum, there was no net secretion of cholesterol, yet the cells continued to secrete 189 μg of apo E per milligram of cell protein. The converse result was obtained with monensin. At a concentration of 1 μ*M*, this agent reduced the secretion of apo E by more than 90 percent without significantly inhibiting cellular protein synthesis. Despite this reduction in apo E secretion, the cells secreted nearly as much cholesterol in the presence of monensin as they did in its absence. Figure 2 shows the effects of varying monensin concentrations on the secretion of cholesterol and ^{35}S–apo E into the medium. At concentrations as low as 0.1 μ*M*, monensin inhibited apo E secretion by 70 percent yet it had no effect on cholesterol secretion.

These experiments show that cholesterol-loaded macrophages can secrete cholesterol at near-normal rates even when secretion of apo E is inhibited by monensin. Conversely, in the absence of a cholesterol-accepting substance, secretion of apo E continues normally, but a net loss of cellular cholesterol does not occur. Thus, although cholesterol and apo E secretion are both stimulated when the cells have accumulated cholesteryl ester, the two substances appear to leave the cells by different mechanisms. Cholesterol is likely to leave the macrophage by transferring from the macrophage plasma membrane to HDL in the medium (*4*, *10*). In contrast, apo E is likely to be secreted through the Golgi apparatus; hence its sensitivity to monensin.

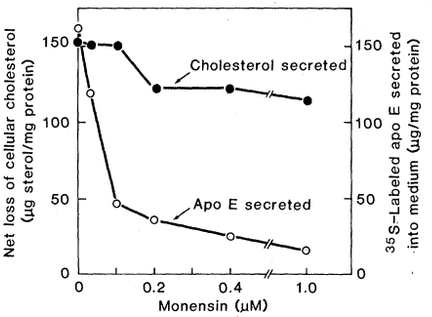

Fig. 2. Dissociation of secretion of cholesterol and apo E in mouse macrophages by monensin. This experimental design was the same as that described in the legend to Table 1 except that the concentration of monensin was varied as indicated.

Since the secretion of apo E is not required for cholesterol secretion, its function must lie elsewhere. We have performed two experiments which suggest that macrophage-produced apo E can form stable complexes with plasma HDL. (i) When the ^{35}S-labeled lipoprotein fraction produced by cholesterol-loaded macrophages was incubated with HDL_3 (density, 1.125 to 1.215 g/ml) and then subjected to agarose electrophoresis, all of the ^{35}S-radioactivity of apo E comigrated with HDL (3.0 cm from origin); in the absence of HDL_3, all of the ^{35}S–apo E migrated more rapidly (4.5 cm from origin). (ii) When incubated in the presence of HDL_3, the macrophage-secreted ^{35}S–apo E was susceptible to precipitation by an antibody to HDL that does not recognize apo E; in the absence of incubation with HDL_3, ^{35}S–apo E was not immunoprecipitated by the antibody.

High-density lipoprotein that contains apo E [so-called HDL_c (*11*)] binds to hepatic LDL receptors with high affinity (*12*). We therefore postulate that the apo E and cholesterol secreted by macrophages bind to HDL and are assembled into HDL_c in the extracellular fluid. This mechanism may serve to transport to the liver the cholesterol that is released from the breakdown of lipoproteins and other cholesterol-containing materials in macrophages. Such "reverse cholesterol transport" (*13*) may play an important role in normal and disease states.

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