2×10^{-9} mole of LTD_4, but only partially blocked (79.1 percent) the decrease of systolic shortening of the myocardial region supplied by the LCx coronary artery. There is a major statistically significant difference between the average response to LTD_4 of both LCx coronary flow and regional shortening before and after FPL administration ($P < .005$), but no such difference for LAD regional shortening.

In the sheep, intracoronary injection of LTD_4 causes local coronary vasoconstriction and myocardial dysfunction. Our observations suggest that ischemia is in part responsible for the impairment of ventricular contraction after LTD_4 injection since the onset of dysfunction occurs concomitantly with decreased coronary artery flow and the contraction pattern then appears characteristic of myocardial ischemia (*15*). The magnitude of coronary flow reduction produced by 2×10^{-9} mole of intracoronary LTD_4 is greater than that required to produce contraction abnormalities on an ischemic basis. Waters *et al.* (*16*) have demonstrated in dogs that a 48 ± 4 percent or greater reduction of coronary flow is associated with both metabolic and mechanical contraction abnormalities. Our studies suggest that LTD_4 causes a negative inotropic effect as well, independent of its effect on coronary blood flow, since intracoronary injection of 2×10^{-9} mole of LTD_4 induced a 14.7 ± 5 percent decrease of systolic shortening in the LCx territory after the administration of FPL 55712 despite lack of significant reduction of coronary flow.

Although the heart and lungs are closely mechanically coupled in the thorax, investigators have long suspected a humoral connection between cardiac and pulmonary function. The recent chemical identification of LTC_4 and LTD_4 produced by lung mast cells and macrophages may provide such a link in selected disease states. Our studies support this possible role for LTD_4. New and sensitive analytical methodology should elucidate the role played by the leukotrienes.

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12. A total of 76 injections was administered to the five sheep. The number of injections of normal saline alone and normal saline with 2×10^{-13}, 2×10^{-12}, 2×10^{-11}, 2×10^{-10}, and 2×10^{-9} mole of LTD_4 were respectively, 8, 3, 4, 4, 4, and 14 before administration of ibuprofen and 9, 4, 4, 6, and 12 afterwards. The order in which the injections were administered was haphazard except that for each animal all injections prior to ibuprofen administration preceded the injections given after ibuprofen. The course of each injection was summarized by the LCx coronary artery flow value, expressed as percentage of the baseline LCx coronary artery flow, at its most extreme departure from the baseline value. The LCx coronary artery flow data were analyzed by an analysis of variance (ANOVA) that adjusted for possible differences in the response of different animals. The experimental design was treated as an incomplete block design with animals as blocks. This analysis ignores any time effect that might be present. The overall *F*-test for differences in the average responses at different doses was determined to be highly significant ($P << .001$); subsequent comparisons of the average responses at particular doses were carried out with *t*-tests based on the estimate of experimental error obtained from the ANOVA. Various models for the dose-response relationship were compared by regression analysis. A linear inverse log dose relationship was adequate. A total of eight injections of LTD_4 (2×10^{-9} mole) were administered to three sheep (one to each sheep before FPL administration and one or more after FPL administration). The resulting data were analyzed by ANOVA. The *F*-test identified significant differences in the average responses before and after FPL administration for LCx coronary artery flow ($P = .004$) and LCx shortening ($P = .005$), but no difference for LAD shortening.
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High-Affinity Choline Transport in Proteoliposomes Derived from Rat Cortical Synaptosomes

Abstract. *Functional high- and low-affinity choline transport processes from rat cortical plasma membranes were reconstituted in phosphatidylcholine bilayer liposomes. The high-affinity choline transporter demonstrated a pharmacological profile and ion dependency that were identical to those of intact synaptosomes. This preparation may be used to further characterize choline transport and, with appropriate supplementation, to investigate the release of acetylcholine in the absence of synaptic vesicles.*

Cholinergic nerve terminals have a high-affinity transporter for choline that subserves acetylcholine synthesis and that is not found in other neurons (*1*, *2*). This transporter appears to be both rate-limiting and regulatory for transmission in the central nervous system under various conditions in vivo (*3*, *4*). High-affinity choline uptake, like other transport systems in the brain, is poorly understood because of the biochemical and morphological complexity of the preparations currently available to study it. This is in contrast to uptake systems in other tissues, such as luminal glucose transport (*5*) or the ion transport in renal medullary cells mediated by Na- and K-dependent adenosinetriphosphatase (*6*), which have been studied extensively with membrane vesicle preparations that allow direct manipulations of the intra- and extracellular milieu. We now describe a preparation of liposomes derived from synaptosomal plasma membranes that exhibit both high- and low-affinity choline uptake activities and is thus amenable to investigation at the molecular level.

Rat cortical synaptosomes were prepared as described (*7*), and suspended in 0.32*M* sucrose (*p*H 7.4) containing 100 μ*M* each of tris-HCl and EDTA. The synaptosomes were frozen and thawed twice, centrifuged at 22,000*g* for 10 minutes, and then suspended in a small volume of buffered sucrose (20 mg of protein per milliliter). Portions of a tissue suspension (50 μl) were added to tubes containing 5 m*M* $MgCl_2$, 0.5 percent sodium deoxycholate, azolectin-derived phosphatidylcholine (ICN) (2 mg/ml), 150 m*M* KCl, and 5 m*M* Hepes buffer (*p*H 7.2). After being sonicated for 30 seconds at room temperature (Braun

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Table 1. Pharmacological characteristics and ionic dependencies of liposomal and synaptosomal [^{3}H]choline uptake. Rat cortical synaptosomes and phosphatidylcholine–plasma membrane liposomes (containing KCl buffer internally) were incubated for 1 minute in the specified external medium at 0° or 25°C with 1 μM [^{3}H]choline (10 Ci/mmole). [^{3}H]Choline uptake was terminated by adding an equal volume of cold NaCl buffer, and total uptake was determined as described. Each value represents the mean ± standard error of four to eight samples.

External medium	Choline uptake (pmole/mg-min)	
	Synaptosomes	Liposomes
NaCl buffer (25°C)	17.4 ± 0.7	23.9 ± 3.2
NaCl buffer (0°C)	3.6 ± 0.5	6.2 ± 1.8
NaCl buffer + hemicholinium-3 (10 μM)	7.0 ± 2.1	12.8 ± 2.1
Na^+ replaced with K^+	4.9 ± 1.5	10.7 ± 0.8
Na^+ replaced with tris-HCl	5.6 ± 0.3	11.9 ± 3.7
Cl^- replaced with SO_4^{2-}	8.2 ± 1.2	14.2 ± 1.4
Cl^- replaced with F^-	4.5 ± 0.4	9.9 ± 1.6
NaCl buffer + gramicidin D (2 μM)	10.9 ± 1.8	8.5 ± 2.1

Sonicator, 100 W), the samples were dialyzed against 10^4 volumes of the sonication medium for 18 hours at 4°C, then 2 hours at room temperature (19° to 21°C). The suspension was centrifuged at 100,000g for 1 hour, and the sediment was suspended in buffered sucrose. The tissue suspension was diluted with 20 volumes of the external medium (150 mM NaCl, 5 mM $MgCl_2$, and 5 mM Hepes, pH 7.2) and then incubated with [^{3}H]choline (4 mCi/μmole, New England Nuclear) and, in some samples, 10 μM hemicholinium-3 (Sigma), 10 μg/ml gramicidin D, or isosmotic replacements for NaCl (KCl, tris-HCl, NaF, or Na_2SO_4). After a 1-minute incubation at 25°C, samples were rapidly chilled in an ice bath, centrifuged for 10 minutes at 45,000g, washed twice, and suspended in 0.01N HCl. The radioactivity of the suspensions was counted in scintillator formula 963 (New England Nuclear). The difference between uptake at 25°C and at 0°C was designated as temperature-dependent choline uptake; this component was found to be osmotically labile, indicating actual uptake and not binding.

No intact synaptosomes were found on electron microscopy after these procedures of freezing, treatment with detergent, and dialysis. Instead, the entire preparation consisted of typical phosphatidylcholine bilayer liposomes, 200 to 300 Å in diameter. The proteoliposomal choline uptake was biphasic with increasing choline concentrations (Fig. 1) and had apparent K_m's of 0.8 μM and 65 μM. These two values correspond to the high- and low-affinity uptake processes observed in intact brain synaptosomes, which have K_m values of about 1.5 μM and 70 μM, respectively (*3*). None of the labeled choline was recovered as acetylcholine or phosphorylcholine. Choline acetyltransferase (E.C. 2.3.1.6) activity was not detected in lysed or intact proteoliposomes (*8*).

The high-affinity uptake of choline by this proteoliposomal preparation was lost if intact synaptosomes were first exposed to hyposmotic shock, if Mg^{2+} ions were removed during detergent treatment, or if tissue was omitted. In addition, the high-affinity choline uptake was reduced if phospholipid concentrations were higher than 5 mg/ml, whereas nonspecific binding and low-affinity uptake of this compound increased dramatically at phospholipid concentrations above this value. Therefore, a phosphatidylcholine concentration of 2 mg/ml was used to maintain optimal high-affinity uptake and to reduce background radioactivity.

At a low choline concentration, the pharmacological profiles of synaptosomal and proteoliposomal choline uptake were similar (Table 1). In the absence of any treatment, liposomes took up choline faster than synaptosomes when activity was expressed per milligram of protein. Temperature-dependent proteoliposomal choline uptake was blocked by treatments that specifically reduce high-affinity transport of this compound (*1*); for example, low concentrations of hemicholinium-3, replacement of Na^+ ions with tris-HCl or K^+ ions, and replacement of Cl^- ions with SO_4^{2-} or F^- ions. Proteoliposomal choline uptake was especially sensitive to K^+ ions, with significant inhibition seen at 10 mM K^+. Previous studies have shown that synaptosomal high-affinity choline uptake is also inhibited by K^+ ions (*3*, *9*); our results indicate that this inhibition is due to a direct action on the membrane. We were not able to increase choline uptake by pretreating liposomes with K^+ ions, as has been reported for intact nerve terminals (*10*, *11*). This finding is consistent with the suggestion that depolarization-induced choline uptake is dependent on prior acetylcholine release (*10*, *11*).

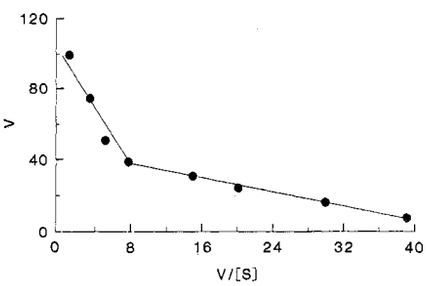

Fig. 1. Eadie-Hofstee plot of liposomal [^{3}H]choline uptake versus substrate concentration. Velocity (V) is expressed as picomoles of choline uptake per milligram of protein per minute. Concentration of substrate (S) is expressed in micromoles per liter. The two lines correspond to K_m's of 0.8 μM and 65 μM choline. Liposomes were prepared containing internal KCl buffer and then suspended for 1 minute at 0° or 25°C in 500 μl of saline buffer plus [^{3}H]choline (0.2 to 100 μM; final specific activity, 0.1 to 50 Ci/mmole). [^{3}H]Choline uptake was terminated by adding an equal volume of ice-cold saline buffer and centrifuging at 40,000g for 10 minutes.

Gramicidin D, which enhances cation exchange across bilayer vesicles (*12*), inhibited high-affinity choline uptake in both synaptosomes and liposomes (Table 1). This result suggests that a membrane potential is necessary for choline uptake into both preparations, since this drug treatment rapidly dissipates the sodium gradient (*12*). Similarly, proteoliposomes in which internal K^+ ions were replaced with Na^+ ions also transported choline more slowly in the presence of the same external Na^+-containing buffer. In normally prepared proteoliposomes (K^+ internal), choline was concentrated about fourfold internally against an external concentration of 1 μM (*13*). Since there was no obvious metabolic energy source for this accumulation, it seems likely that either (i) the energy was provided by the membrane potential or (ii) choline taken up by the liposomes was subsequently bound and rendered inactive. Although we cannot at present rule out the second possibility, our results with Na^+ loading of proteoliposomes suggest that the membrane potential is important for both transport and accumulation of high-affinity choline. The fourfold choline accumulation probably underestimates the accumulation in cholinergically derived proteoliposomes, since this value is derived from a cortical proteoliposome preparation where only a small fraction of the terminals are cholinergic.

Despite the high specific activity of the proteoliposomal choline uptake, only about 20 percent of the total synaptosomal choline uptake was recovered in this reconstituted system. Whether this incomplete recovery is due to the inver-

sion of phospholipid vesicles, or to inactivation or loss of the transporter molecules during reconstitution, is unclear.

The high-affinity choline transporter is the sole supplier of choline for acetylcholine synthesis in the isolated terminal (*9*). In contrast, the low-affinity choline uptake process is found in virtually all cells and presumably subserves phosphorylcholine and subsequently phospholipid synthesis (*14*). Our results show that these two uptake systems can be reconstituted simultaneously into proteoliposomes and, in particular, that the high-affinity transporter retains kinetic and pharmacological properties that are similar to those seen in intact synaptosomes. This transport occurs in the absence of choline acetyltransferase activity, even though kinetic observations in intact terminals suggest that this enzyme is physically coupled to high-affinity uptake (*3, 9, 15*).

γ-Aminobutyrate and glutamate transport activities have also been described in membrane preparations derived from synaptosomes (*16*). However, these preparations resulted from the resealing of hyposmotically lysed terminals, in which we found no high-affinity, hemicholinium-3–sensitive, Na^+-dependent choline uptake. A liposome preparation derived from hyposmotically lysed synaptosomal membranes that retained choline uptake has been described (*17*); however, this uptake was not characterized pharmacologically or kinetically as high-affinity transport. For example, hemicholinium-3 only slightly inhibited choline uptake, and no dependence on external sodium or chloride ions was demonstrated.

The proteoliposomal preparation we have described appears to be appropriate for isolating and characterizing the high-affinity choline transporter, as well as for studying other neuronal transport mechanisms. We have found that the proteoliposomes take up serotonin at a low concentration in a sodium-dependent manner. In addition, we have shown in preliminary experiments that this preparation, when supplemented internally with acetylphosphate, coenzyme A, choline acetyltransferase, and phosphotransacetylase, releases newly synthesized acetylcholine when depolarized with K^+ in a calcium-dependent manner. The preparation may therefore also be useful for studying nonexocytotic release mechanisms.

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Endotoxin-Stimulated Opioid Peptide Secretion: Two Secretory Pools and Feedback Control in vivo

Abstract. *Small doses of endotoxin evoked a dramatic biphasic response of opioid peptide secretion into blood in sheep. The first phase began within minutes and coincided with a brief hypertensive response to endotoxin well before the appearance of fever or hypotension. The ratio of* β*-endorphin to* β*-lipotropin fell abruptly at the onset of the second phase of release, suggesting early depletion of a pool rich in* β*-endorphin and subsequent emergence of a pool rich in unprocessed precursor. The concentration of cerebrospinal fluid opioids increased tenfold during the second phase. Naloxone administration augmented endotoxin-induced opioid secretion in both early and late phases, suggesting a short-loop feedback regulation of stress-induced endorphin secretion.*

The pressor response to intravenously administered naloxone during endotoxin-induced hypotension in animals, and in some clinical cases of septic shock, suggests a role for endogenous opioids in mediating shock resulting from infection (*1*). Although pyrogens activate the pituitary-adrenal axis (*2*) and hence would be expected to cause release of β-endorphin (β-EP) (*3*), no direct evidence is available regarding the dynamics of opioid peptide secretion in relation to endotoxin-induced hyperthermia or hypotension. A primary role for endogenous opioids in producing vital sign responses to endotoxin might be indicated if peptide release precedes vital sign changes, or a secondary role if vital sign changes precede peptide release.

To characterize endorphin secretory dynamics in endotoxin fever, we used a model that permits rapid atraumatic sampling of blood simultaneously from the carotid artery, jugular vein, and sagittal sinus in alert, unanesthetized, partially restrained sheep (*4*). In addition, in 13 of 14 experiments, cerebrospinal fluid (CSF) was sampled from a lateral ventricle through an implanted cannula (*5*). All animals were tame, acclimated to the testing laboratory, and had free access to food and water at all times. Blood pressure was monitored through the carotid artery line, and temperature was recorded from a rectal thermocouple.

One group (seven sheep) received intravenous bolus injections of endotoxin (*Escherichia coli* serotype 055:B5; 450 ng/kg) (*6*); a second group (three sheep) was given endotoxin plus naloxone (an intravenous bolus of 0.1 mg/kg injected with the endotoxin, then an infusion of 0.02 mg/kg per hour) (*7*); and a control group (four sheep) was given 0.9 percent saline only. In each experiment, lactated Ringer solution was infused into the jugular vein at a rate equal to the rate of blood withdrawal. Plasma and CSF samples were assayed for immunoreactive β-EP (iβ-EP) (*8*). The molecular weight profile of iβ-EP was obtained by G-50 gel chromatography of plasma extracts (*9*).

Figure 1A depicts vital sign changes in relation to plasma and CSF concentrations of iβ-EP in a representative animal treated with endotoxin. A composite diagram of results obtained in plasma of the seven sheep given endotoxin is also shown (Fig. 1B). Temperature in these sheep first rose to a level two standard deviations above baseline at a mean ± standard error (S.E.M.) of 30.0 ± 7.7 minutes after endotoxin administration. A brief period of hypertension always preceded hyperthermia, presumably reflecting vasoconstriction during the so-called "chill" phase long recognized as an immediate consequence of endotoxin administration (*10*). Hypotension followed the transient hypertension, after

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