Characterization and localization of neutral sphingomyelinase in bovine adrenal medulla

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Abstract Homogenates of bovine adrenal medullae hydrolyzed exogenous sphingomyelin at 4.3 ± 1.6 nmol · mg^{-1} · min^{-1} and 97% of this sphingomyelinase activity was sedimentable at 110,000 *g*. The sphingomyelinase had a broad pH optimum centered at pH 7. Enzymatic activity was maximal with 80 μM added Mn^{2+}; Mg^{2+} supported less than half maximal activity and both Ca^{2+} and EDTA inhibited activity. No activity was detected in the absence of Triton X-100. Response to detergent was biphasic with dose-dependent stimulation from 0.02% to 0.05% Triton X-100 followed by inhibition with increasing concentrations of detergent. Activity in response to detergent was also modulated by protein concentration. Sphingomyelinase activity was associated with a plasma membrane–microsomal fraction. Phosphatidylcholine was not hydrolyzed under optimal conditions for sphingomyelin hydrolysis and a variety of other conditions. Neutral-active sphingomyelinase activity in adrenal medulla was similar in magnitude to that observed in other non-neural bovine tissues. This study demonstrates the presence of a potent neutral-active sphingomyelinase in a plasma membrane–microsomal fraction of bovine adrenal medulla. This enzyme may be involved in membrane fusion and lysis during catecholamine secretion through its ability to alter membrane composition. — **Bartolf, M., and R. C. Franson.** Characterization and localization of neutral sphingomyelinase in bovine adrenal medulla. *J. Lipid Res.* 1986. **27:** 57–63.

Supplementary key words lipid metabolism • sphingolipid metabolism • exocytosis • catecholamine • chromaffin granule • plasma membrane • phospholipase C • Triton X-100 • Mn^{2+}-dependent

Two types of sphingomyelinases have been reported: a ubiquitous lysosomal enzyme which is soluble, acid-active and ion-independent; and a non-lysosomal enzyme which is membrane-associated, neutral-active, Mg^{2+}-dependent, and is found in brain and to a lesser extent in other tissues (1). The lysosomal enzyme is presumably involved in lipid digestion within the phagocytic vacuole (2), while the neutral-active, membrane-associated enzyme is thought to degrade sphingomyelin in a controlled fashion to influence membrane fluidity by altering the phosphatidylcholine/sphingomyelin ratio (3) or by indirectly affecting the cholesterol content of membranes (4).

Recent studies in adrenal medulla indicate that membrane phospholipid perturbations may contribute to the exocytotic release of catecholamines. Thus, aggregated chromaffin granules from adrenal medulla fuse upon addition of *cis*-unsaturated fatty acids (5). Such fatty acids could be generated in situ by the direct action of phospholipase A_2 or the concerted action of phospholipase C and diglyceride lipase. However, neutral-active, Ca^{2+}-dependent phospholipase A_2 activity is largely suppressed in tissue homogenates and extracts of adrenal medulla (6). During thin-layer chromatographic analyses of medullary lipids, we noted significant amounts of diglyceride. Therefore, we examined adrenal medulla for the presence of phospholipases C which could generate diglyceride. Recently, we described a cytosolic phosphatidylinositol-specific phospholipase C that binds to chromaffin granule membranes in a Ca^{2+}-dependent manner (7). We have not detected nonspecific phospholipase C activity, but have found and now report the presence of a potent neutral-active sphingomyelinase that is almost totally membrane-associated. Like previously reported neutral-active sphingomyelinases, this enzyme appears to be specific for hydrolysis of sphingomyelin and requires detergent for expression of activity in vitro (1). But unlike previously described neutral-active sphingomyelinases, the adrenal medullary enzyme is maximally active with added Mn^{2+}. Since plasma and secretory granule membranes of adrenal medulla are enriched in sphingomyelin (8, 9), this sphingomyelinase may contribute to membrane phospholipid perturbations associated with the exocytotic release of catecholamines.

METHODS

Preparation of tissue homogenates and subcellular fractions

Bovine adrenal glands and other bovine tissues were obtained fresh from the slaughter house and placed in ice until use. The following procedures were done at 0–4°C unless indicated otherwise.

In experiments to determine sphingomyelinase in tissue

homogenates and sphingomyelinase distribution in the soluble and membrane fractions of homogenates, 1 g wet weight of tissue was minced and homogenized in 10 ml of 25 mM bis-Tris, pH 7.0, 50 mM NaCl. Debris was removed by sedimentation at 600 *g* for 15 min. The resulting supernatant fraction was frozen and thawed, and was sedimented at 110,000 *g* for 90 min to yield soluble (supernatant) and membrane (resuspended pellet) fractions.

In subcellular localization studies, medullae were cut into small pieces and then macerated in 0.3 M sucrose by two 3-sec bursts of a Waring blender (10 volumes per g wet weight of tissue). This was followed by homogenization with three strokes of a motor-driven Potter-Elvehjem homogenizer. After removal of debris by sedimentation at 500 *g* for 10 min, the homogenate was fractionated into granule and membrane fractions by the following procedures. After an initial centrifugation of the homogenate at 20,400 *g* for 30 min, the supernatant was carefully decanted for use in preparing the plasma membrane–microsome fraction described below. The 20,400-*g* pellet was resuspended in two-thirds of the starting volume of 0.3 M sucrose and subjected to two additional sedimentations at 10,900 *g* and 7,700 *g* for 30 min each to yield a final pellet enriched in chromaffin granules, lysosomes, and mitochondria (granule fraction, **Table 1,** 10, 11). Before enzymatic and catecholamine analyses the granule fraction was resuspended in 25 mM bis-Tris, pH 7.0, 50 mM NaCl and was frozen and thawed to ensure organelle lysis. The plasma membrane–microsome fraction was prepared by sedimenting the 20,400 *g* supernatant fraction described above, at 110,000 *g* for 90 min (Table 1). The resulting pellet was resuspended by homogenization in 25 mM bis-Tris, pH 70, 50 mM NaCl, and frozen and thawed prior to enzymatic analyses; or it was resuspended in 40% sucrose (w/w) for application to sucrose step gradients (12).

Sphingomyelinase assay

Sphingomyelinase activity was measured using sphingomyelin labeled with ^{14}C in the N-methyl moiety of choline. The sphingomyelin in $CHCl_3$–CH_3OH 6:1 (v/v) was evaporated to dryness under N_2 and then resuspended in deionized water by two 10-sec sonications. Reaction mixtures in a total volume of 0.5 ml contained 110 nmol of sphingomyelin (12,000 cpm) and appropriate buffer, metal ions and Triton X-100 (w/v) as indicated in the individual figures and table. Following incubation at 37°C for the times specified in the figures, reactions were stopped with 1.5 ml of $CHCl_3$–CH_3OH 2:1 (v/v), followed by the addition of 0.5 ml of 2.0 M KCl and 0.5 ml of $CHCl_3$. A portion (0.75 ml) of the aqueous phase (upper phase) was removed for liquid scintillation counting to determine release of radioactive phosphocholine. Each data point is the average of duplicate determinations. In all experiments where subcellular distribution of sphingomyelinase was investigated, conditions were determined to assure linearity of the assay. In general, the neutral-active, membrane-associated sphingomyelinase of adrenal medulla did not lose activity with storage at −20°C, or with repeated freezing and thawing over periods of several weeks' use.

Other assays

Catecholamines were determined colorimetrically. A perchloric acid extract (7%, final concentration) was brought to pH 7.8 with 0.2 M potassium phosphate. Potassium ferricyanide was added to a final concentration of 0.044% and mixed thoroughly. After exactly 5 min at room temperature, the absorbance was read at 485 nm (13). β-Glucuronidase (14), cytochrome c oxidase (15, 16), and acetylcholinesterase (17) were determined by established procedures. The release (18) of phosphate by glucose-6-phosphatase was measured by the method of Chen, Toribara, and Warner (19). Protein was determined by the Coomassie brilliant blue method using bovine serum albumin as standard (20).

MATERIALS

Sphingomyelin (bovine, [^{14}C]CH_3-labeled choline, 40–60 mCi/nmol) was purchased from New England Nuclear

TABLE 1. Sphingomyelinase and marker enzyme activities in subcellular fractions from bovine adrenal medulla[a]

	Homogenate	Granule Fraction	Membrane Fraction
Sphingomyelinase	5.5 ± 0.3	1.7 ± 0.5 (0.3)[b]	18.1 ± 3.1 (3.3)
Catecholamines	0.8 ± 0.1	1.7 ± 0.3 (2.1)	0.1 ± 0.0 (0.1)
β-Glucuronidase	1.1 ± 0.3	2.3 ± 0.5 (2.1)	1.1 ± 0.2 (1.0)
Cytochrome c oxidase	58.8 ± 8.3	85.3 ± 14.8 (1.5)	13.2 ± 4.6 (0.2)
Acetylcholinesterase	15.3 ± 3.5	5.9 ± 3.0 (0.4)	38.5 ± 16.3 (2.5)
Glucose-6-phosphatase	4.3 ± 0.5	2.1 ± 0.4 (0.5)	14.5 ± 2.5 (3.4)

[a] Specific activities are in nanomoles of substrate converted per min per mg protein, except catecholamines which are in micromoles per mg protein. Values represent the average of three separate determinations ± SD.

[b] Numbers in parentheses are purification factors and are the specific activity of each fraction relative to the specific activity of the homogenate.

(Boston, MA) and nonradioactive sphingomyelin (bovine) was purchased from Supelco, Inc. (Bellefonte, PA).

RESULTS

Bovine adrenal medullae were homogenized under hypoosmotic conditions (50 mM NaCl) and the homogenate was frozen and thawed to ensure organelle lysis. The resulting homogenate hydrolyzed sphingomyelin at 4.3 ± 1.6 nmol · mg^{-1} · min^{-1} (n = 3) when assayed under optimal conditions of pH 7.0, 1 mM Mn^{2+}, and 0.07% Triton X-100. Sedimentation of the homogenate at 110,000 *g* for 90 min generated soluble and membrane fractions with specific activities of 0.2 ± 0.0 and 11.5 ± 3.0 nmol · mg^{-1} · min^{-1} (n = 3), respectively. The resuspended membrane fraction contained 97.4 ± 0.6% (n = 3) of the recoverable activity and was therefore utilized in subsequent characterization studies. All specific activities were determined under conditions that assured linearity of the assay.

This membrane-associated sphingomyelinase had a broad pH optimum centered at approximately pH 7 (**Fig. 1**). EDTA at 1 mM abolished activity throughout the pH range indicating the presence of cation-dependent sphingomyelinase and the absence of cation-independent, lysosomal sphingomyelinase in the membrane fraction. Mn^{2+} was more effective than Mg^{2+} in supporting activity in the optimal pH range. The ratio of Mg^{2+}- to Mn^{2+}-supported activity increased with increasing pH from 0.15 at pH 5 to 1.05 at pH 9.

The effects of Mg^{2+}, Mn^{2+}, and Ca^{2+} on membrane-associated sphingomyelinase at pH 7 are shown in **Fig. 2A.** Maximal activity was noted at 80 μM Mn^{2+}, with 50% of maximal activity at less than 10 μM Mn^{2+}; there was no further change in activity to 1.0 mM Mn^{2+}. At pH 7, Mg^{2+} was less effective in supporting activity than Mn^{2+}; the ratio of Mg^{2+}- to Mn^{2+}-supported activity increased with increasing metal ion concentrations from 0.22 at 10 μM to 0.45 at 1.0 mM. Ca^{2+} inhibited basal activity. The simultaneous presence of optimal concentrations of both Mn^{2+} and Mg^{2+} did not support sphingomyelinase activity above that observed with Mn^{2+} alone. Moreover, maximal activity with Mn^{2+} (80 μM) was decreased by added Mg^{2+} in a dose-dependent manner (Fig. 2B), suggesting competition for the same metal binding site. As can be seen in the figure, sphingomyelinase activity with optimal Mn^{2+} was reduced by Mg^{2+} in a dose-dependent fashion until it approached maximal Mg^{2+}-supported activity (dashed line). Ca^{2+} was less effective than Mg^{2+} at diminishing Mn^{2+}-supported activity.

Sphingomyelin hydrolysis was proportional to time of incubation at lower protein concentrations, but deviations from linearity were noted at higher protein concentrations (**Fig. 3A**). Thus, with 13.3 μg of membrane protein per incubate, hydrolysis was linear for at least 60 min, while with 26.6 μg and 53.2 μg it was linear for less than 40 min and 20 min, respectively. Sphingomyelin hydrolysis was not proportional to protein concentration (Fig. 3B). This was due in part to the dependence of sphingomyelinase activity on protein-Triton X-100 ratios (**Fig. 4**). The response of sphingomyelinase to Triton X-100 was biphasic; activity was first stimulated by Triton X-100 in a dose-dependent manner, and then inhibited as the detergent concentration was increased. No activity was detectable in the absence of detergent. As Triton X-100 concentrations were increased above those shown in Fig. 4, activity was inhibited further. The concentration of Triton X-100 required to support maximal activity increased with increasing protein concentration and the concentration range of Triton X-100 required for optimal activity broadened somewhat with increasing protein concentration. In Fig. 3, activity was maximal with 53.2 μg of protein (and higher protein) in the presence of 0.1% Triton X-100, but this detergent concentration resulted in sphingomyelinase activities 10% and 30% below maximal with

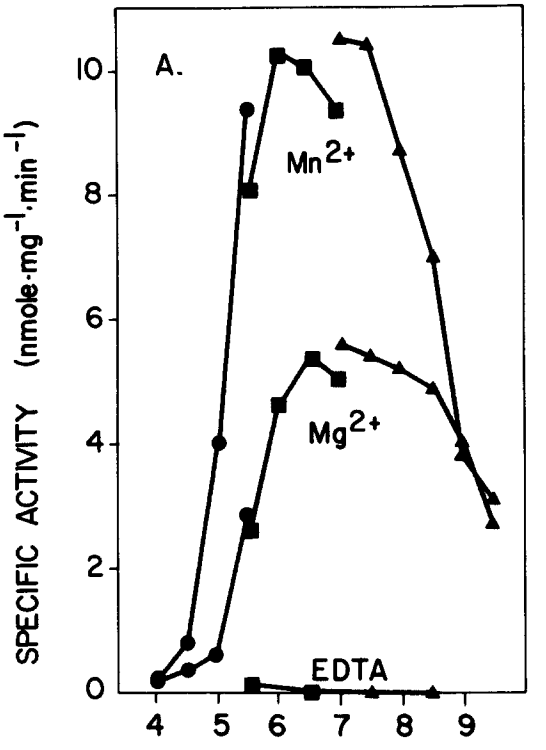

Fig. 1. Sphingomyelin hydrolysis as a function of pH (abcissa) by the membrane faction (110,000 *g* pellet) of an adrenal medullary homogenate. Sphingomyelinase was assayed as described in Methods under the following conditions; Mn^{2+} profile: 1 mM Mn^{2+}, 0.07% Triton X-100, 32.0 μg of protein, 30-min incubation; Mg^{2+} profile: 1 mM Mg^{2+}, 0.07% Triton X-100, 32.0 μg of protein, 60-min incubation; EDTA profile: 1 mM EDTA, 0.07% Triton X-100, 37.6 μg of protein, 60-min incubation. The following buffers were used: (●) 50 mM acetate, (■) 50 mM bis-Tris, and (▲) 50 mM glycylglycine.

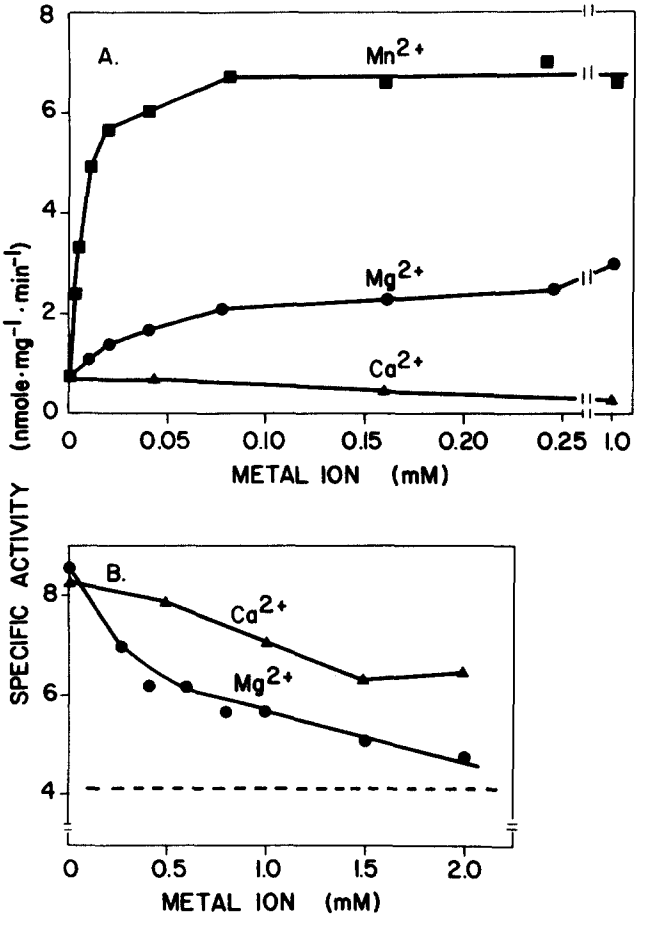

Fig. 2. Sphingomyelin hydrolysis as a function of divalent cation concentration by the membrane fraction (110,000 *g* pellet) of an adrenal medullary homogenate. A. Sphingomyelinase was assayed as described in Methods under the following conditions: 50 mM HEPES, pH 7.0, 0.07% Triton X-100, 46.9 μg of protein, 40-min incubation (0.01–1.0 mM Mn^{2+}) or 80-min incubation (2.5 and 5.0 μM Mn^{2+}, all Mg^{2+} and Ca^{2+}), and divalent cations as indicated in the figure. B. Sphingomyelinase was assayed as described in Methods in the presence of 0.07 mM Mn^{2+}, 50 mM HEPES, pH 7.0, 0.07% Triton X-100, 26.6 μg of protein, 30-min incubation with the addition of either Mg^{2+} or Ca^{2+} as indicated in the figure. The dashed line is sphingomyelinase activity with 2 mM Mg^{2+} only.

26.6 μg and 13.3 μg of protein, respectively. This resulted in the reduced activity at lower protein concentrations in Fig. 3B. The reduced activity at higher protein concentrations may have been due to the more extensive substrate hydrolysis at higher protein concentrations resulting in lack of sufficient substrate to maintain linear kinetics. When the data of Figs. 3A and 3B were plotted as specific activity versus % hydrolysis (Fig. 3C), it was apparent that specific activity decreased as % hydrolysis increased above approximately 8%. This decrease in activity was not due to the hydrophobic product, since addition of exogenous ceramide did not reduce activity (data not shown). The data points for 13.3 μg of protein plotted in Fig. 3C showed reduced activity since for this amount of protein 0.1% Triton X-100 was inhibitory (Fig. 4).

To determine the subcellular localization of the membrane-associated sphingomyelinase, adrenal medullary homogenates in 0.3 M sucrose were fractionated into granule and membrane fractions by differential sedimentation. The specific activity of sphingomyelinase decreased in the granule fraction and increased in the 110,000-*g* membrane fraction (Table 1). Since the granule fraction was enriched in markers for chromaffin granules, lysosomes, and mitochondria, and the membrane fraction was either unchanged (lysosomal marker) or reduced in these markers, neutral-active sphingomyelinase was not associated with these organelles. The plasma membrane marker, acetylcholinesterase, and the microsomal marker, glucose-6-phosphatase, were enriched in the membrane fraction and reduced in the granule fraction. Thus, the neutral-active sphingomyelinase of adrenal medulla was

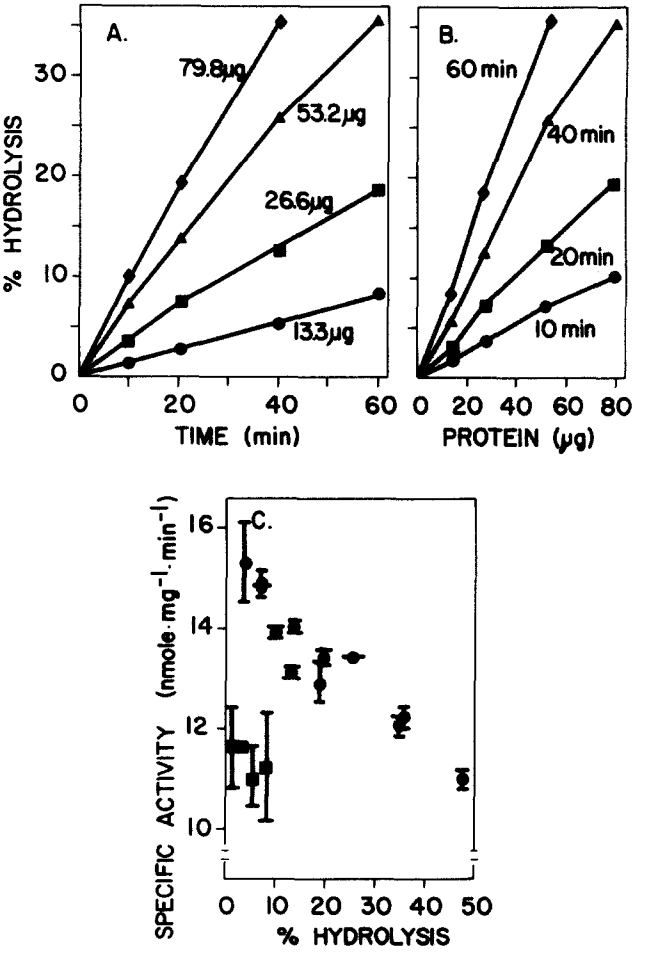

Fig. 3. Sphingomyelin hydrolysis as a function of time of incubation and protein concentration by the membrane fraction (110,000 *g* pellet) of an adrenal medullary homogenate. Sphingomyelinase was assayed as described in Methods under the following conditions: 50 mM HEPES, pH 7.0, 0.1% Triton X-100, 1 mM Mn^{2+}, and μg of protein and times of incubation as indicated in the figure. In C the data points from A and B were plotted as specific activity (nmol · mg^{-1} · min^{-1}) vs % hydrolysis; (■) 13.3 μg of protein and (●) all other data points.

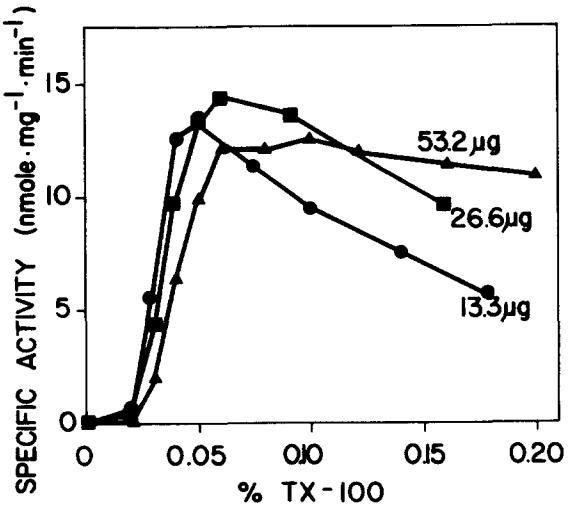

Fig. 4. Sphingomyelin hydrolysis as a function of Triton X-100 concentration by the membrane fraction (110,000 *g* pellet) of an adrenal medullary homogenate. Sphingomyelinase was assayed as described in Methods under the following conditions: 50 mM HEPES, pH 7.0, 1 mM Mn^{2+}, (●) 13.3 µg of protein, 60-min incubation, (■) 26.6 µg of protein, 20-min incubation, or (▲) 53.2 µg of protein, 10-min incubation, and Triton X-100 as indicated in the figure.

associated primarily with plasma membranes and/or microsomes. Further fractionation of the membrane fraction on sucrose step gradients did not result in separation of plasma membranes from microsomes as previously reported (12), but did enhance sphingomyelinase specific activity to 130 nmol·mg^{-1}·min^{-1}, almost 24 times homogenate sphingomyelinase activity. This fraction was similarly enriched in both acetylcholinesterase and glucose-6-phosphatase.

DISCUSSION

These results demonstrate the presence of a potent, neutral-active, Mn^{2+}(Mg^{2+})-dependent sphingomyelinase in the plasma membrane-microsomal fraction of bovine adrenal medulla. Neutral-active sphingomyelinase activity in homogenates of bovine adrenal medulla (4.3 ± 1.6 nmol·mg^{-1}·min^{-1}) is similar in magnitude to that of rat or human (Triton X-100 solubilized) brain, but significantly greater than that of rat or human liver, kidney, or spleen (21, 22). This enzyme, associated with 24-fold enriched membranes, has a specific activity (130 nmol·mg^{-1}·min^{-1}) that is several-fold greater than that of a partially purified enzyme from infantile human brain (23) and is 43-fold that of a plasma membrane fraction from rat liver (24). Thus, sphingomyelinase activity in bovine adrenal medulla is significantly greater than that previously reported in tissues other than brain from both rat and human sources. Although the adrenal medulla is of neural origin, the relatively high activity in this bovine tissue probably reflects a species difference, since the specific activity of sphingomyelinase in homogenates of non-neural bovine tissues is similar to that of adrenal medulla (data not shown).

The optimal pH range (pH 6–8, Fig. 1) for adrenal medullary sphingomyelinase activity is slightly broader and somewhat more acidic than those previously observed (2, 24, 25). This enzyme resembles the previously reported neutral active, Mg^{2+}-dependent sphingomyelinases in that it is inhibited by EDTA and stimulated by Mg^{2+} and Mn^{2+}, but not Ca^{2+}. However, it differs from previously reported sphingomyelinases in its sensitivity to Mn^{2+}. Human brain sphingomyelinase was less sensitive to Mn^{2+} than to Mg^{2+} and required 2–2.5 mM Mg^{2+} for maximal activity (22). Although rat liver sphingomyelinase was 16% more active with Mn^{2+} than Mg^{2+}, both metal ions were needed in relatively high concentrations for maximal activity (4 and 40 mM, respectively), and the range of maximal activity with Mn^{2+} was relatively narrow (24). In contrast, the bovine adrenal medullary sphingomyelinase reported here is maximally active (at pH 7.0) with 80 µM added Mn^{2+}, while the same concentration of Mg^{2+} supports only 30% of this activity (Fig. 2). At optimal metal ion concentrations (1 mM, Fig. 1), the relative abilities of Mn^{2+} and Mg^{2+} to support activity are pH dependent. Thus, near neutral pH, Mn^{2+} supports twice as much activity as Mg^{2+}. As the pH decreases Mn^{2+} becomes relatively more effective than Mg^{2+} in supporting activity; with increasing pH, both cations become equally effective in supporting activity. The lack of additivity of Mg^{2+}- and Mn^{2+}-supported activities and the reduced ability of Mn^{2+} to support activity in the presence of excess Mg^{2+} (Fig. 2B) suggest that both metal ions act at the same site. Moreover, the inability of Ca^{2+} to support activity implies specific metal ion requirements rather than merely nonspecific charge effects on the substrate. Collectively, these data indicate that Mn^{2+} is the preferred metal ion in vitro for the neutral-active, membrane-associated sphingomyelinase of bovine adrenal medulla. However, because tissue Mg^{2+} levels are at least 100-fold greater than those of Mn^{2+}, Mg^{2+} may be the physiologically relevant cation.

The neutral-active sphingomyelinase from adrenal medulla, like other neutral-active sphingomyelinases (21, 23, 25), is not active in vitro in the absence of added detergent (Fig. 4). Like infantile human brain sphingomyelinase (23), adrenal medullary sphingomyelinase is first stimulated and then inhibited by increasing concentrations of Triton X-100. However, the adrenal medullary sphingomyelinase is much more sensitive to small changes in Triton X-100 concentrations below 0.05% and less sensitive at higher detergent–protein ratios than is infantile human brain sphingomyelinase (23). The biphasic response to Triton X-100 is due to interactions of detergent with both substrate and enzyme (26). Below 0.013%

Triton X-100, sphingomyelin is dispersed as bilayered liposomes and is not hydrolyzed. Thus, in Fig. 4 there is no hydrolysis in the absence of Triton X-100. Above 0.013% Triton X-100, mixed micelles of detergent and sphingomyelin are formed and hydrolysis occurs in a detergent-dependent fashion since the amount of sphingomyelin in micellar form increases with increasing detergent concentration, in effect increasing the hydrolyzeable sphingomyelin concentration. Above a Triton X-100–sphingomyelin ratio of approximately 4 (about 0.06% Triton X-100 in Fig. 4) the detergent forms pure Triton X-100 micelles and sphingomyelinase activity is not further enhanced. In fact, further increases in detergent concentration inhibit sphingomyelinase, presumably by inactivating the enzyme. At Triton X-100 concentrations below 0.05% (in Fig. 4), the enhancement of sphingomyelinase activity by Triton X-100 is inversely related to protein concentration. Presumably, protein interacts with Triton X-100 and reduces its ability to form mixed micelles with sphingomyelin. At higher concentrations of Triton X-100, reduction of optimal sphingomyelinase activity by superoptimal detergent concentrations is also inversely related to protein concentration. This probably also results from the ability of protein to bind Triton X-100 and thereby reduce its effective concentration. The specific substrate-detergent ratios required for optimal hydrolysis of sphingomyelin in vitro may reflect a hydrolytic requirement for specific physical forms of membrane phospholipid in vivo.

The only thorough subcellular localization study of neutral-active sphingomyelinase was done with rat liver, where the enzyme was localized primarily to plasma membranes and secondarily to microsomes (24). The bovine adrenal medullary sphingomyelinase is also membrane-bound; more than 97% was found in the 110,000-*g* pellet. This sphingomyelinase did not cosediment with chromaffin granules, lysosomes, or mitochondria since the relatively low sphingomyelinase activity present in the granule fraction can be accounted for by plasma membrane or microsomal contamination (Table 1). Attempts to separate plasma membranes from microsomes on a sucrose step gradient by a procedure used for the isolation of plasma membranes from adrenal medulla (12) were not successful. In our hands the 32–36% sucrose interface was equally enriched in plasma membranes (acetylcholinesterase) and microsomes (glucose-6-phosphatase), whereas Meyer and Burger (12) observed a greater enrichment of acetylcholinesterase than glucose-6-phosphatase in this fraction. The significantly different procedures used by Meyer and Burger (12) and ourselves to prepare samples for sucrose gradient fractionation may have been responsible for this discrepancy. Meyer and Burger (12) used a Percoll gradient fraction from a 25,000-*g* pellet of an adrenal medullary homogenate, whereas we utilized a 110,000-*g* pellet from a 20,400-*g* supernatant of an adrenal medullary homogenate. However, sucrose gradient sedimentation is a good purification step since it yields a fraction enriched almost 24-fold in sphingomyelinase over the homogenate. Thus, the neutral-active sphingomyelinase of adrenal medulla is localized in a plasma membrane–microsome-enriched fraction, which is in agreement with results in rat liver (24).

The bovine adrenal medullary sphingomyelinase did not hydrolyze phosphatidylcholine under optimal conditions for sphingomyelin hydrolysis and a variety of other conditions (data not shown). Moreover, unlike the partially purified lysosomal sphingomyelinase from rabbit alveolar macrophages (27), the bovine adrenal medullary sphingomeylinase did not hydrolyze the phospholipids in human myelin (data not shown). However, in view of the dependence of phospholipase activities on the physicochemical state of phospholipid substrates (28, 29), it is possible that under suitable in vitro assay conditions or in situ the enzyme described herein could hydrolyze other phospholipids.

The phospholipid composition of adrenal medullary plasma membranes and chromaffin granule membranes is similar and consists of approximately 14–15% sphingomyelin, 29–36% phosphatidylcholine, and 32–34% phosphatidylethanolamine (8, 9). Thus, these membranes are enriched in sphingomyelin. Sphingomyelin differs from phosphatidylcholine and phosphatidylethanolamine in its hydrophilic region, where it is able to form hydrogen bounds, while phosphatidylcholine and phosphatidylethanolamine are not. In the hydrophobic region, the average length of hydrocarbon chains in sphingomyelin is greater and their degree of unsaturation is less than in phosphatidylcholine and phosphatidylethanolamine. In addition, due to differences in chain length between the hydrocarbon region of sphingosine and the amide-linked acyl chain, more than 50% of sphingomyelin molecules are very asymmetric (30). Thus, changes in the proportions of these three phospholipids in membranes may alter membrane structure and dynamics. Since fusion of plasma membranes with chromaffin granule membranes, and subsequent membrane lysis is observed during catecholamine secretion in adrenal medulla (31), alterations in membrane properties through changes in sphingomyelin content could play a role in the exocytotic process. The neutral-active, $Mn^{2+}(Mg^{2+})$-dependent, membrane-associated sphingomyelinase reported here for the first time in adrenal medulla could be involved in secretion-related sphingomyelin alterations.

This investigation was supported by an NIH Grant A1-18644. Richard C. Franson is the recipient of a Research Career Development Award, NIH HL-00561.

Manuscript received 12 June 1985.

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