Journal of Bioenergetics and Biomembranes, Vol. 14, Nos. 5/6, 1982

Localization of Endogenous ATPases at the Nerve Terminal

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Received May 3, 1982; revised June 29, 1982

Abstract

We have investigated the localization of a set of intrinsic ATPase activities associated with purified synaptic plasma membranes and consisting of (a) a Mg^{2+}-ATPase; (b) an ATPase active at high concentrations of Ca^{2+} in the absence of Mg^{2+} (Ca_H-ATPase); (c) a Ca^{2+} requiring Mg^{2+}-dependent ATPase (Ca + Mg)-ATPase, stimulated by calmodulin (Ca-CaM-ATPase); (d) a Ca^{2+}-dependent ATPase stimulated by dopamine (DA-ATPase); and (e) the ouabain-sensitive (Na + K)-ATPase. The following results were obtained: (1) All ATPases are largely confined to the presynaptic membrane; (2) the DA-, (Ca + Mg)-, (Ca-CaM)-, and (Na + K)-ATPases are oriented with their ATP hydrolysis sites facing the synaptoplasm; (3) the Mg- and Ca_H-ATPases are oriented with their ATP hydrolysis sites on the junctional side of the presynaptic membrane and are therefore classified as ecto-ATPases of as yet unknown function.

Key Words: ATPase; Ca-Mg-ATPase; calmodulin; synaptic junctions; pre- and postsynaptic membranes; ectoenzymes; enzyme localization; nerve membranes.

Introduction

Depolarization of the presynaptic membrane constitutes the initial event leading to the release of neurotransmitters from storage sites within chemical synapses. This event causes a voltage-dependent efflux of K^+ from the terminal together with an influx of Na^+ and subsequently of Ca^{2+} ions into the terminal. According to the well-documented "calcium hypothesis" (Katz and Miledi, 1969), it is the net increase in the internal Ca^{2+} ion concentration that serves as the trigger for the release of neurotransmitter molecules (Llinas and

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0145-479X/82/1200-0527$03.00/0

Heuser, 1977; Blaustein *et al.*, 1978; Kelly *et al.* 1979). Clearly, once this period of activity has ended, the ionic composition of the nerve terminal must be restored to its resting, steady-state condition. This is achieved, in part, through the activity of transport adenosine triphosphatases (ATPases) present within the presynaptic plasma membrane (Barritt, 1981).

By definition, ATPases are enzymes able to catalyze the hydrolysis of ATP to ADP and inorganic phosphate. Transport ATPases, also known as ion pumps, use the free energy change of this hydrolysis to transport ions against an electrochemical gradient across the membrane bilayer (Barritt, 1981; Stekhoven and Bonting, 1981; Wilson, 1978; Dahl and Hokin, 1974). The transport ATPases documented for nerve tissue include the well-characterized (Na + K)-ATPase[2] or Na^+-pump (reviewed by Robinson and Flashner, 1979, and Hobbs and Albers, 1980), which is used as a standard marker enzyme for brain and other plasma membrane preparations (Gurd *et al.*, 1974; Jones and Matus, 1974; Babitch *et al.*, 1976; DeBlas and Mahler, 1978), and a recently described (Ca + Mg)-ATPase or Ca^{2+}-pump (DeBlas and Mahler, 1978; Trotta and deMeis, 1978; Sobue *et al.*, 1979; Robinson, 1981; Gill *et al.*, 1981).

Nontransport ATPases have also been located at the nerve terminal. These include the activities associated with cytoskeletal proteins such as myosin (Puszkin and Kochwa, 1974; Berl, 1975) or tubulin (White *et al.*, 1980); in addition, there is evidence for Ca/Mg-ATPases in synaptic vesicle and other neurotransmitter storage granule preparations (Rothlein and Parsons, 1979; Michaelson *et al.*, 1980; Toll and Howard, 1980; Apps *et al.*, 1980). In the former, the free-energy change of ATP hydrolysis is probably used for promoting force-generating interactions, and in the latter, for maintaining the electrochemical gradient of protons required for transmitter uptake into the vesicles.

We have recently described (Sorensen and Mahler, 1981) several ATPase activities associated with a purified synaptic membrane preparation from rat brain including: (a) a Mg^{2+}-requiring activity; (b) a low-affinity Ca^{2+}-requiring, Mg^{2+}-independent activity; (c) a high-affinity Ca^{2+}-stimulated, Mg^{2+}requiring ATPase which is enhanced by the addition of the ubiquitous Ca^{2+}-binding protein calmodulin (CaM) (Cheung, 1980; Klee *et al.*, 1980); and (d) a neurotransmitter-stimulated, Ca^{2+} + Mg^{2+}-requiring

[2]Abbreviations: ATPases, Mg-ATPase, Mg^{2+}-requiring; Ca_H-ATPase, low-affinity Ca^{2+}-requiring, Mg^{2+}-independent; (Ca + Mg)-ATPase, high-affinity Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase; Ca-CaM-ATPase, high-affinity Ca^{2+}-requiring, CaM-activated, Mg^{2+}-dependent ATPase; DA-ATPase, Ca^{2+}-requiring, dopamine- (cathecholamine) stimulated; (Na + K)-ATPase, Na^+ plus K^+-requiring; CaM, calmodulin; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*',*N*'-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; LDH, lactate dehydrogenase; SDH, succinate dehydrogenase; SA, specific activity units per milligram protein; unit (U, micromoles substrate turned over per minute.)

ATPase activity. To gain further insight into the possible roles of these ATPases in neuronal function, we now document the intrasynaptic localization of these enzymes by monitoring their activities in several well-characterized preparations derived from nerve-terminal membranes.

Experimental

Materials

Male Sprague–Dawley rats, 30–40 days old, were purchased from Harlan Industries, Indianapolis, Indiana. *N*-2-Hydroxethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), ATP (disodium salt), ethyleneglycol-bis-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), dopamine-HCl (DA); dithiothreitol (DTT), saponin, NADH (Tris-base), ouabain, phenazine methosulfate, dichlorophenolindophenol, and sodium lauryl sulfate (SDS) were purchased from Sigma Chemical Co., St. Louis, Missouri. Sucrose (special enzyme grade) was from Schwarz-Mann, Orangeburg, New York. Digitonin was from Sigma Chemical Co. and was twice recrystallized in absolute EtOH (Kun *et al.*, 1979). Calmodulin (CaM) was prepared from bovine brain by the method of Watterson *et al.* (1980). All other chemicals were of reagent grade or better.

Preparation of Subcellular Fractions

Synaptosomes were prepared by the method of Hajos (1975), collected from the 0.8 M sucrose layer, and usually equilibrated in KCl or NaCl media as described by Blaustein and co-workers (Blaustein *et al.*, 1978; McGraw *et al.*, 1980). Synaptic membranes (SM) were prepared by the method of Salvaterra and Matthews (1980), and synaptic plasma membranes (SPM) according to Gurd *et al.* (1974). Synaptosomes (Hajos, 1975) were further fractionated on a five-step gradient as described by Babitch *et al.* (1976). Postsynaptic membranes (PSM) with attached postsynaptic densities were isolated as described by Ratner and Mahler (1979), and P_3B_2, a light microsomal membrane preparation, was isolated as described by DeBlas and Mahler (1978). Modifications of these procedures are described in the legends to the tables.

Enzyme Assays

Lactate dehydrogenase (LDH) was usually assayed in a reaction mixture containing 50 mM potassium phosphate, pH 7.4, 400 μM sodium pyruvate, and 80 mM NADH in a total volume of 1.0 ml, preincubated for 10 min at 30°C, and the reaction was started by the addition of membrane suspension. Activity was determined by following the rate of NADH oxidation at 340 nm.

Modified reaction mixtures used for measuring the LDH activity of some membrane preparations are indicated in the table legends.

Succinate dehydrogenase (SDH) was usually assayed in a reaction mixture containing 50 mM potassium phosphate, pH 7.4, 40 mM sodium succinate, 0.5 mM phenazine methosulfate, 1.5 mM KCN, and 50 μM dichlorophenolindophenol in a total volume of 1.0 ml, preincubated for 10 min at 37°C, and the reaction was started by the addition of membrane suspension. Activity was measured spectrophotometrically by following the rate of change at 600 nm. Modified reaction mixtures used for measuring the SDH activity of some preparations are indicated in the table legends.

Endogenous ATPase activity was measured in a reaction mixture containing 50 mM HEPES, pH 7.6, 1 mM DTT, 0.5 mM EGTA, and 5 mM NaN_3 in a total volume of 500 μl. Various ATPase activities are defined and measured by the following additions to the basic assay mixture (Sorensen and Mahler, 1981):

(a) Mg-ATPase: addition of 6 mM $MgCl_2$ and 2 mM ouabain;
(b) (Ca + Mg)-ATPase: as in (a) plus 0.5 mM $CaCl_2$; activity is defined as (b)–(a);
(c) Ca-CaM-ATPase: as in (b) plus 5 μg CaM; activity is defined as (c)–(a);
(d) Ca_H-ATPase: addition of 5 mM $CaCl_2$ and 2 mM ouabain;
(e) DA-stimulated ATPase: addition of 6 mM $MgCl_2$, 2 mM ouabain, and 5 mM $CaCl_2$; activity is defined as the difference between those in the presence and absence of 400 μM dopamine-HCl, 200 μM pargyline;
(f) (Na + K)-ATPase: addition of 6 mM $MgCl_2$, 135 mM NaCl, and 20 mM KCl; activity is defined as the difference between those in the presence and absence of 2 mM ouabain.

Reaction mixtures were preincubated for 10 min at 37°C and the reactions started by the addition of 6 mM ATP (disodium salt). After 5 min at 37°C, the reactions were stopped by the addition of 1.5 ml of 3.5% (w/v) SDS. Inorganic phosphate was measured spectrophotometrically using the ammonium molybdate:HCl system described by Peterson (1978). The modified reaction mixtures used for assaying the ATPase activities of some preparations are indicated in the table legends.

Protein was determined as described by Markwell *et al.* (1978).

Results

ATPase Activity of Isolated Synaptosomes. Five endogenous ATPase activities were selected for this study of their intraterminal localization. The

first approach used isolated, purified synaptosomes. In the design of these experiments we followed a protocol used by Blaustein and co-workers (Blaustein *et al.*, 1978; McGraw *et al.*, 1980) for the study of Ca^{2+}-sequestering sites within the nerve terminal. They showed that the detergent saponin could be used to partially disrupt the plasma membrane, allowing normally impermeable substances, such as ATP and other charged effector molecules, easy access to the internal environment of the nerve terminal, without significantly altering its morphological structures. Similarly, digitonin has been used to increase the permeability of the plasma membrane selectively, without affecting the structure of the internal organelles (Kun *et al.*, 1979; Fiskum *et al.*, 1980). To determine the suitability of either of these detergents for use in the study of synaptosomal ATPase activities, we first tested both on the ATPase activities of isolated synaptic membranes in order to establish possible activating or inhibitory effects, and then determined their ability to permeabilize the synaptosomal plasma membrane.

The effect of saponin and digitonin on the ATPase activities of SM are shown in Table I. Both have little effect on the Mg-ATPase and Ca_H-ATPase activities of these membranes. High concentrations (0.25 mg/ml) of digitonin specifically enhance (Ca + Mg)-ATPase activity by 80%, with a small activation (~20%) of DA- and (Na + K)-ATPase activities. Low concentration (0.05–0.10 mg/ml) of saponin enhance DA-, (Ca + Mg)-, and (Na + K)-ATPase activities by 50%.

Next, we tested saponin and digitonin for their effects on the permeability of the synaptosomal plasma membrane. After detergent treatment, the synaptosome suspensions were separated into particulate (pellet) and soluble (super) fractions by centrifugation, and the extent of membrane permeabilization was determined by following the distribution of the marker enzymes SDH (mitochondria) and LDH (synaptoplasm) into these fractions. The rationale was that relatively intact synaptosomes should have less of the LDH in the soluble fraction than lysed synaptosomes, the membranes of which have been totally disrupted, a treatment that liberates synaptoplasmic enzymes into the soluble fraction after centrifugation. Increasing the permeability of synaptosomes should result in an increase in marker activity in the pellet fraction. In these experiments SDH (not shown) was concentrated in the pellet fraction regardless of the detergent concentration used. This was due to the presence of contaminating mitochondria in this synaptosome preparation, the high SDH activity of which serves to overshadow any activity due to the sequestered synaptosomal mitochondria.

From the observation (not shown) that there was a substantial increase (>10 fold) in the LDH activity in the supernatant fraction upon treatment of intact synaptosomes with high concentrations of detergent, we selected this activity as a marker for increasing the permeability of the synaptosomal membrane. Digitonin (0.05–0.25 mg/ml) did not significantly alter the

Table I. Effects of Detergents on ATPase Activity[a]

Detergent	ATPase, specific activity[b]				
	Mg	Ca_H	DA	Ca + Mg	Na + K
Saponin					
0 mg/ml	362	307	82	204	1135
	(100)	(100)	(100)	(100)	(100)
0.05	428	250	115	309	1759
	(118)	(81)	(140)	(151)	(155)
0.10	414	251	120	334	1742
	(114)	(82)	(146)	(164)	(153)
0.25	370	294	60	320	1300
	(102)	(96)	(73)	(157)	(115)
0.50	290	270	33	160	1130
	(80)	(88)	(90)	(78)	(100)
Digitonin					
0 mg/ml	370	301	88	165	1170
	(100)	(100)	(100)	(100)	(100)
0.05	467	335	81	121	1050
	(126)	(111)	(92)	(73)	(90)
0.10	393	266	66	257	1315
	(105)	(88)	(75)	(156)	(112)
0.25	393	224	107	297	1385
	(105)	(74)	(122)	(180)	(118)

[a]Synaptic membranes (SM) were prepared as described by Salvaterra and Matthews (1980). ATPase activities were measured as described in Experimental in the absence and presence of various amounts of saponin or digitonin. Digitonin was twice recrystallized from 100% EtOH (Kun *et al.*, 1979). Mg: Mg-ATPase; Ca_H: low-affinity Ca-ATPase measured in the absence of Mg^{2+} ion; DA: dopamine-stimulated, calcium-requiring ATPase; Ca + Mg: high-affinity (Ca^{2+} + Mg^{2+})-ATPase; Na + K: (Na^+ + K^+)-, ouabain-sensitive ATPase. See text and Sorensen and Mahler (1981) for definition and measurement of these activities.

[b]Specific activity reported as mU/mg: mean of two experiments. Values in parentheses are calculated relative to values in the absence of detergents set equal to 100%.

distribution of LDH activity or of protein found in the soluble and membrane fraction. However, addition of saponin to 0.10 mg/ml led to an increase of LDH specific activity in the particulate fraction, while 0.25 mg/ml caused a significant shift in the distribution of this activity into the supernatant fraction. Based on these preliminary experiments we selected 0.10 mg/ml saponin for studying the effects of the permeabilization of the presynaptic plasma membrane.

We also tested several methods that had been used previously to lyse synaptosomes for their suitability in studying the effect of complete disruption of the synaptosome on its ATPases. As before, we determined the extent of synaptosomal intactness by means of the distribution of LDH activity into particulate and soluble fractions collected after lysis of the synaptosomes (Table II). We observed that when synaptosomes were disrupted by the lysis medium described by Blaustein *et al.* (1978), only 30% of the total recovered

Table II. Effects of Lysis Conditions on Disrupting Synaptosomes[a]

	Protein		LDH	
	Total[b]	Recovery[c] (% of total)	SA[d]	Recovery[e] (% of total)
Control		(99)[f]		(87)[g]
Whole	2.44	—	813	—
Super	0.64	26	736	27
Pellet	1.78	74	709	73
H_2O		(106)		(70)
Whole	2.18	—	962	—
Super	0.45	19	2811	86
Pellet	1.87	81	106	14
H_2O plus KCl		(109)		(82)
Whole	2.89	—	642	—
Super	1.24	39	1081	89
Pellet	1.91	61	90	11
Tris		(122)		(77)
Whole	1.76	—	751	—
Super	0.49	23	1813	87
Pellet	1.65	77	81	13
Tris plus KCl		(109)		(78)
Whole	2.39	—	666	—
Super	1.19	46	960	91
Pellet	1.42	54	75	9
Lysis		(108)		(87)
Whole	4.60	—	426	—
Super	2.58	52	244	37
Pellet	2.39	48	449	63
Lysis plus KCl		(112)		(107)
Whole	3.97	—	491	—
Super	2.19	49	463	48
Pellet	2.27	51	475	52

[a]"KCl-synaptosomes" were prepared as follows: Particles were prepared in 0.8 M sucrose according to Hajos (1975), diluted 2:3 with cold distilled H_2O to 0.32 M sucrose, and equilibrated on ice with KCl (McGraw *et al.*, 1980) by the slow addition, with constant stirring, of half the volume of (3 ×) KCl medium to yield (final concentration) 50 mM HEPES, pH 7.6, 145 mM KCl, 5 mM NaCl, 6 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, and 0.21 M sucrose. The suspension was centrifuged at 10,000 × *g* for 20 min and resuspended in the KCl medium just described but without sucrose. For separation of soluble (super) and particulate (pellet) fractions, suspensions were centrifuged for 5 min at 20,000 × *g*. After the initial centrifugation, the pellet was resuspended (2 ml per brain) in either cold distilled H_2O (H_2O), 5 mM Tris-base, pH 8.1 (Tris), or the media described by McGraw *et al.* (1980) (lysis) consisting of 50 mM HEPES, pH 7.6, 6 mM $MgCL_2$, 0.5 mM EGTA, and 1 mM DTT. After incubation for 15 min on ice, aliquots of the suspension were restored to KCl media (plus KCl). Whole, Pellet, and Super fractions were collected and assayed for SDH and LDH activity with no modifications to the reaction mixtures. Control—synaptosomes kept intact on ice for the period required to expose experimental samples to the various lysis media. SDH was also assayed but remained virtually completely (~90%) in the pellet fraction under all conditions tested.

[b]Total milligrams of protein recovered.

[c]Percent of total protein recovered assuming a recovery of 100%.

[d]specific activity, mU/mg.

[e]Percent of total units recovered assuming a total recovery of 100%.

[f]Percent of total protein recovered.

[g]Percent of total units recovered.

LDH activity was found in the supernatant. However, when H_2O or 5 mM Tris, pH 8.1, was used to disrupt the synaptosomes, 90% of the total LDH activity was recovered in the supernatant. Returning the synaptosomal suspension to the KCl medium ("plus KCl") after lysis but before centrifugation did not affect the recovery of LDH activity in the fractions; however, it did cause an increase in the amount of protein recovered in the supernatant and, correspondingly, a lowering of the specific activity of LDH. We therefore chose to use H_2O for the lysis of the synaptosomes.

We then used two separate approaches for studying the distribution of endogenous ATPase activities at the nerve terminal. In the first (Table III) we examined whether they were located in the membranes or soluble (synaptoplasmic) fraction of synaptosomes after increased disruption of their membranes. In these experiments no attempt was made to maintain intactness of synaptosome structure after separating the two fractions, so that their total activity could be measured and directly compared with the total activity initially present in the unfractionated synaptosomes. Again, LDH activity was used to monitor the extent of intactness of the synaptosomes. When assayed in KCl media, little ATPase activity and, also, little LDH activity, was found in the supernatant fraction collected from intact synaptosomes. Disruption of the synaptosomal plasma membrane by saponin treatment or lysis resulted in LDH activity being liberated into the soluble fraction, while all ATPase activities remained largely (>90% of total recovered) in the particulate fraction. These results suggest that the ATPase activities under study are localized in the membranes or membraneous organelles of the nerve terminal. They do not rule out the possibility that, while LDH activity is truly soluble, other activities may be localized in synaptoplasmic organelles, such as synaptic vesicles, that become attached to the synaptoplasmic membrane and will therefore appear localized in the particulate fraction after centrifugation of the disrupted synaptosomes. However, this is not a very likely eventuality. Specifically, although synaptic vesicles have been observed to remain associated with synaptic membranes isolated from lysed synaptosomes (Wang and Mahler, 1976), the proportion of attached vesicles is relatively small. Also, synaptic vesicles can be isolated from the synaptoplasm of synaptosomes lysed in a manner similar to that used in these experiments (Whittaker *et al.*, 1964; DeLorenzo and Freedman, 1978). These observations suggest that synaptic vesicles, and any other small internal organelles, will follow the LDH activity into the supernatant.

In the second set of experiments we wanted to determine the sidedness of the ATP hydrolysis sites, i.e., whether they are situated on the synaptoplasmic face of the presynaptic membrane, as contrasted with its junctional face, or the postsynaptic membrane. In these experiments (Table IV) synaptosomes were either kept intact, rendered permeable (Perm), or lysed following

Table III. Membranous Versus Synaptoplasmic Location of ATPases[a]

	Protein		LDH		ATPase							
					Mg		DA		Ca + Mg		Na + K	
	Total	Recovery (% of total)	SA	Recovery (% of total)	SA	Recovery (% of total)	SA	Recovery (% of total)	SA	Recovery (% of total)	SA	Recovery (% of total)
"KCl" Medium												
Intact		(107)		(80)		(78)		(?)		(101)		(81)
Whole	5.97	—	344	—	165	—	−2	—	30	—	137	—
Super	1.20	19	255	19	27	4	−3	0	−9	0	−11	0
Pellet	5.16	81	260	81	143	96	11	100	35	100	129	100
Perm		(111)		(77)		(73)		(96)		(76)		(67)
Whole	5.38	—	357	—	171	—	29	—	67	—	300	—
Super	1.46	24	423	42	47	10	6	6	−2	0	−4	0
Pellet	4.50	76	192	58	133	90	31	94	61	100	239	100
Lysed		(112)		(77)		(65)		(127)		(61)		(60)
Whole	3.77	—	437	—	167	—	17	—	66	—	222	—
Super	1.40	33	806	89	34	12	7	11	−7	0	5	1
Pellet	2.83	67	51	11	127	88	25	88	54	100	174	99

[a]Synaptosomes equilibrated in KCl media were prepared as described in the legend to Table II. After centrifugation, the pellets were resuspended in either (a) KCl media (intact); (b) KCl containing 0.1 mg/ml saponin (perm); or (c) H_2O, followed by returning the suspension to KCl medium (lysed). Whole, pellet, and super fractions were then collected (see Table II). LDH activity was measured in the presence of KCl medium containing 400 μM sodium pyruvate and 80 μM NADH. ATPase activity was measured in the presence of KCl medium containing: for (a) Mg-ATPase, no further additions; (b) for DA-ATPase, plus 5 mM $CaCl_2$; this activity is the difference in the absence and presence of 400 μM dopamine and 200 μM pargyline; (c) for (Ca + Mg)-ATPase, plus 0.5 mM $CaCl_2$; this activity is the difference in the activity of (c) minus (a); and for (d) (Na + K)-ATPase, no further additions except 2 mM ouabain; this activity is the difference in the activities measured in the presence and absence of ouabain. Values are reported as described in Table II. A negative value for a specific activity indicates an activity *less* than base line [activity (a)]. When synaptosomes were isolated and their activities measured in NaCl medium (similar to KCl except for 145 mM NaCl plus 5 mM KCl), all values and distributions were similar to those obtained in KCl medium. Data shown are for one of two experiments.

Table IV. Junctional Location of ATPasers[a]

		SDH		LDH		ATPase: Mg		Ca_H		DA		Ca + Mg		Na + K	
		SA	% Change	SA	% Change	SA	% Change	SA	% Change	SA	% Change	SA	% Change	SA	% Change
KCl medium															
Intact	(a)	19 ± 6	100	125 ± 61	100	156 ± 72	100			13 ± 13	100	4 ± 4	100	28 ± 7	100
	(b)	24 ± 8	100	117 ± 67	100			110 ± 43	100						
Perm	(a)	25 ± 4	132	572 ± 250	458	164 ± 74	105			46 ± 30 (31)	354 (238)	58 ± 39 (39)	1450 (975)	210 ± 102 (140)	750 (500)
	(b)	26 ± 6	108	596 ± 351	509			83 ± 53 (101)	75 (92)						
Lysed	(a)	26 ± 8	137	650 ± 303	520	174 ± 70	112			76 ± 58	585	40 ± 8	1000	214 ± 26	764
	(b)	18 ± 7	75	614 ± 278	525			146 ± 49	133						
NaCl medium															
Intact	(a)	20 ± 1	100	140 ± 43	100	157 ± 52	100			7 ± 7	100	12 ± 14	100	36 ± 22	100
	(b)	18 ± 2	100	116 ± 52	100			140 ± 59	100						
Perm	(a)	23 ± 1	115	485 ± 162	346	148 ± 53	94			45 ± 30 (20)	643 (429)	72 ± 45 (48)	600 (400)	306 ± 131 (204)	850 (567)
	(b)	19 ± 3	106	473 ± 163	408			111 ± 79 (135)	79 (96)						
Lysed	(a)	22 ± 10	110	616 ± 298	440	156 ± 72	99			46 ± 38	657	60 ± 39	500	190 ± 92	528
	(b)	20 ± 6	111	558 ± 262	481			144 ± 73	103						

[a]Synaptosomes (Hajos, 1975) were collected in 0.8 M sucrose and diluted with an equal volume of (2 ×) KCl or NaCl media (final concentration as in Table III) which was added dropwise, with constant stirring, at 0–4°C (final concentration 0.4 M sucrose). The equilibrated synaptosomes were then treated in the following manner: (a) intact, no further treatment; (b) perm, slow addition to the synaptosome suspension of 0.1 volume of the appropriate ionic media containing 0.4 M sucrose and 1.0 mg/ml saponin (final concentration 0.1 mg/ml), followed by incubation of ≥30 min prior to assaying enzyme activities; or (c) lysed, diluted 1 part synaptosome suspension to 3 parts of the appropriate ionic media (final concentration 0.1 M sucrose). Although Blaustein and co-workers (Blaustein *et al.*, 1978; McGraw *et al.*, 1980) report that synaptosomes remain intact after equilibration in high-salt media in the absence of sucrose, we observed that 0.3–0.4 M sucrose plus the high-salt media was necessary to ensure good intactness of the synaptosomes. Lowering the sucrose concentration below 0.3 M resulted in an enhancement of measured LDH activity by 2–5-fold, indicating an increase in the lysis of the synaptosomes even in the presence of high salt. LDH and all ATPase activities were assayed in reaction mixtures consisting of the appropriate media with the required additions as described in Table III. SDH activity was determined in a reaction mixture consisting of the appropriate ionic media plus 40 mM sodium succinate, 0.5 mM phenazine methosulfate, 1.5 mM KCN, and 50 μM dichlorophenolindophenol. To ensure that no further lysis of the intact and perm synaptosomes occurred during the assay of enzyme activity, the reaction mixtures of these synaptosomes contained 0.32–0.4 M sucrose. The reaction mixtures for the perm synaptosomes also contained 0.1 mg/ml saponin during all enzyme assays. SA: specific activity, mU/mg; (a) synaptosomes equilibrated in media containing 6 mM $MgCl_2$; (b) synaptosomes equilibrated in media without 6 mM $MgCl_2$ (used to assay Ca_H-ATPase activity); percent change in SA is expressed relative to values for intact synaptosomes set equal to 100%. Numbers in parentheses represent values corrected for the stimulation or inhibition of these activities in isolated membranes in the presence of saponin (see Table I). $n = 4$.

equilibration of the synaptosomes in KCl or NaCl media, and then assayed for SDH, LDH, and ATPase activities in reaction mixtures identical to the final incubation media in which the synaptosomes had been suspended originally. To do this, special care was taken to guard against further lysis of the intact and saponin-treated synaptosomes during separation and enzyme assays. Blaustein *et al.* (1978) reported that equilibration of such treated synaptosomes in KCl media permitted their study without damaging the membrane. In our hands, we observed that the LDH activity of the synaptosomes increased two- to threefold when the sucrose concentration was reduced below 0.32 M during the equilibration step, indicating that it had resulted in their partial lysis. In contrast, maintenance of the sucrose concentration between 0.32–0.4 M during equilibration resulted in no change in LDH activity, and therefore we used this strategy in our protocols. The results (Table IV) show increases in LDH activity of 4.8- and 5.2-fold in the saponin-treated and lysed synaptosomes, respectively, when assayed in KCl media, and 3.7- and 4.6-fold, respectively, when assayed in NaCl media. Disruption of the membrane did not result in any significant increase in the Mg- and Ca_H-ATPase activities, whereas the DA-, (Ca + Mg)-, and (Na + K)-ATPase activities were stimulated 5–10-fold after disruption or opening of the synaptosomal membrane by lysis or saponin treatment. If corrections are made for the stimulation of DA-, (Ca + Mg)-, and (Na + K)-ATPase activities due to exposure to 0.10 mg/ml saponin alone (see Table I), these ATPase activities were still enhanced 4–5-fold by treating the synaptosomes with saponin.

ATPase Activities of Synaptosomal Subfractions. In this series of experiments, we examined the intrasynaptosomal location of the various ATPase activities under study by determining their presence in several well-characterized subsynaptic membrane preparations. We used SDH activity as a marker for their mitochondrial content, and (Na + K)-ATPase activity as a plasma membrane marker (Stekhoven and Bonting, 1981).

First we examined the ATPase activities in the synaptic plasma membrane (SPM) preparation described by Gurd *et al.* (1974). This preparation consists of presynaptic membranes with relatively few postsynaptic attachments. The results (Table V) show that ATPase activities are concentrated in fractions of the density gradient lighter than those containing the highest levels of SDH. Maximum activity for each of the ATPases, including the plasma membrane marker (Na + K)-ATPase, was found in the lightest (0.32/0.6 M sucrose interface) fraction of the gradient.

Second, lysed synaptosomes prepared according to Hajos (1975) were subjected to fractionation on the five-step discontinuous sucrose density gradient described by Babitch *et al.* (1976). Synaptosomes prepared in this manner [i.e., in media not containing EDTA or EGTA to chelate endogenous Ca^{2+} (Cotman and Taylor, 1972)] would be expected to contain attached

Table V. Distribution of ATPase in SPM[a]

Fraction	Protein (mg)	SDH (mU × mg^{-1})	ATPase (mU × mg^{-1})					
			Mg	Ca_H	DA	Ca + Mg	Ca-CaM	Na + K
Synaptosomal particulate	59.2	110	280	142	112	110	153	1,096
A′-II, 0.32/0.6 interface	7.0	3.3	485	391	103	171	244	1,970
A-II, 0.6/0.8 interface	10.5	34	347	258	75	146	173	1,840
B-II, 0.8/1.0 interface	7.2	94	266	139	91	83	130	1,058
C-II, 1.0/1.3 interface	5.4	112	172	69	53	47	62	668
D-II, pellet	4.4	80	68	81	35	45	45	365
Recovery (%)	(58)	(31)	(63)	(85)	(39)	(59)	(55)	(71)

[a]Synaptic plasma membranes (SPM) were prepared as described by Gurd *et al.* (1974) following their method II which includes 1 mM phosphate/0.1 mM EDTA in all buffers. A minor modification was made in the lysis of P_2. The washed pellet, collected from the Ficoll gradient, was resuspended in 5 volumes of 1 mM phosphate/0.1 mM EDTA for lysis. After separation by centrifugation in a discontinuous sucrose density gradient, all interfaces were collected, diluted with cold H_2O, and centrifuged at 78,000 × *g* for 30 min. Material was stored in distilled H_2O. Protein, SDH, and ATPase assays were as described in Experimental. Nomenclature for the different fractions is that in the original paper (Gurd *et al.*, 1974). Activities are expressed as mU/mg. Numbers in parentheses indicate the percent of total protein or enzyme activity recovered from the sucrose gradient.

postsynaptic specializations (Whittaker *et al.,* 1964; Cotman and Taylor, 1972; Jones and Matus, 1974; Cohen *et al.,* 1977), and, correspondingly, the membranes collected from the sucrose density gradient should consist of both pre- and postsynaptic membranes connected at the cleft region. That the postsynaptic specializations have indeed remained attached in this preparation is suggested by the observation that the plasma membrane marker (Na + K)-ATPase was now concentrated in a fraction banding in more concentrated sucrose than in the previous experiment, indicating that the membranes were of higher buoyant density. This result would be predicted on the basis of the retention of the postsynaptic density, a proteinaceous organelle, subjacent to the membrane. The results (Table VI) show that SDH activity was again largely found in the heaviest gradient fractions, whereas the ATPase activities were found in lighter fractions. Maximum activities for each of the ATPases were concentrated in the 0.6/0.8 and 0.8/0.95 M sucrose interfaces of the gradient except for the DA-ATPase activity which appeared localized in the two light fractions in the first experiment, suggesting its presence in synaptic vesicles.

Next, we studied a membrane preparation designed to consist largely of postsynaptic membranes with attached postsynaptic densities (Ratner and Mahler, 1979). In the preparation of these membranes, the starting material [synaptic membranes prepared according to Salvaterra and Matthews (1980)] is subjected to two salt extractions, followed by extraction with EGTA. After these treatments, the extracted membranes are sonicated before the material is applied to a discontinuous sucrose density gradient. Preliminary experiments (not shown) indicated that the salt and EGTA extractions of SM caused little ATPase activity to be removed from the membranous material. However, upon sonication of the extracted membranes, both the amount of protein and the recovery of all ATPase activities were equipartitioned between the supernatant and pellet fractions collected after this treatment. The specific activities determined for the ATPases distributed between these two fractions were also similar. Therefore, sonication did not specifically remove ATPase activity from the membranes, but rather resulted in a dispersal of membranes of similar composition between the supernatant and pellet fractions.

We next studied the distribution of the ATPase activities in the final sucrose gradient used in the preparation of PSM, a heavy membrane fraction collected from a 1.5/2.0 M sucrose interface. Table VII shows that the ATPase activities were concentrated in the 0.32/1.0 and 1.0/1.5 M sucrose interfaces whereas SDH activity was concentrated in the 1.0/1.5 and 1.5/2.0 M sucrose interfaces. Thus the ATPases appear associated with presynaptic and junctional membranes.

Finally, P_3B_2, a light microsomal membrane subfraction, which has been

Table VI. Distribution of ATPase After Separation by Five-Step Sucrose Density Gradient[a]

			ATPase					
	Protein (mg)	SDH (mU × mg^{-1})	Mg	Ca_H	DA	Ca + Mg	Ca-CaM	Na + K
			Experiment 1					
Synaptosomal particulate	48.3	100	235	197	47	130	137	678
Gradient fraction								
0.32/0.6	0.9	3.1	172	113	114	64	76	310
0.6/0.8	2.8	2.3	420	274	139	164	144	1,312
0.8/0.95	3.6	22	291	181	84	143	165	1,040
0.95/1.1	6.4	35	184	161	71	101	114	652
Recovery (%)	(59)	(27)	(51)	(47)	(85)	(45)	(44)	(59)
			Experiment 2					
Synaptosomal particulate	55.0	107	198	132	21	95	101	618
Gradient fraction								
0.32/0.6	0.3	ND	101	79	23	78	56	188
0.6/0.8	1.6	3.2	264	173	6	193	231	909
0.8/0.95	3.3	35	288	146	0	171	222	1,327
0.95/1.1	8.4	57	203	125	42	113	129	809
Pellet	26.9	53	120	73	16	52	52	338
Recovery (%)	(74)	(40)	(60)	(53)	(69)	(63)	(65)	(65)

[a]Synaptosome isolated by the method of Hajos (1975) were subjected to fractionation on a five-step discontinuous sucrose gradient as described by Babitch *et al.* (1976). The synaptosomes were collected in 0.8 M sucrose, slowly diluted with cold distilled H_2O to 0.4 M sucrose, and centrifuged at 38,000 × *g* for 20 min. The resulting pellet was resuspended in H_2O (5 ml per brain) for lysis, placed on ice for 15 min, and centrifuged again as above. The pellet was resuspended in 5 ml of 0.32 M sucrose and applied to a discontinuous sucrose gradient (Babitch *et al.*, 1976) consisting of 8 ml 0.6 M sucrose, 8 ml 0.8 M sucrose, 10 ml 0.95 M sucrose, and 8 ml 1.1 M sucrose and centrifuged at 68,000 × *g* for 90 min. The material from the interfaces was collected, diluted with cold distilled H_2O, and centrifuged at 78,000 × *g* for 30 min. All material was stored in distilled H_2O. Protein, ATPase, and SDH assays were done as described in Experimental.

Table VII. Distribution of ATPase Activities in PSM and Fractions of the Sucrose Density Gradient[a]

			ATPase					
	Protein	SDH	Mg	Ca_H	DA	Ca + Mg	Ca-CaM	Na + K
Particulate after sonication	57.0	75	240	211	39	87	127	1,113
Fraction A, 0.32/1.0 interface	8.2	3.8	305	257	43	159	161	1,481
Fraction B, 1.0/1.5 interface	19.2	165	276	253	35	82	79	1,049
PSM, 1.5/2.0 interface	1.75	167	104	194	8	63	83	580
Recovery (%)	(51)	(82)	(58)	(61)	(47)	(60)	(41)	(52)

[a]Postsynaptic membranes with attached postsynaptic densities (PSM) were prepared as described by Ratner and Mahler (1979). After separation of the extracted and sonicated material by discontinuous sucrose density gradient fractionation, all interfaces were collected, diluted with cold distilled H_2O, and centrifuged at 78,000 × *g* for 30 min. Pellets were stored in distilled H_2O. Protein, SDH, and ATPase assays were done as described in Experimental. Protein is expressed in milligrams; ATPase activities, in mU × mg^{-1}.

shown to be enriched in receptor function for several neurotransmitters (DeBlas and Mahler, 1978; Near and Mahler, 1981), was prepared and the membranous material, collected at the interfaces of each discontinuous sucrose density gradient, was assayed for ATPase activity (Table VIII); SDH and (Na + K)-ATPase activities were not measured in this experiment but were taken from the original paper of DeBlas and Mahler (1978). These studies had indicated that SDH activity was largely confined to the pellets (fractions P_3C and P_3B_3) collected from the final two sucrose gradients used in preparing P_3B_2, whereas (Na + K)-ATPase activity was localized in lighter fractions (P_3B in the first gradient; P_3B_2 and P_3B_2 in the second). All ATPase activities measured were localized in the same fractions as the (Na + K)-ATPase activity in the second gradient, with the possible exception of the (Ca + Mg)-ATPase and the (Ca-CaM)-ATPase. DeBlas and Mahler (1978) observed (Ca + Mg)-ATPase activity of P_3B_1 to be much higher than that determined in the present experiment but found it to be localized in the same fractions as the (Na + K)-ATPase activity. The low recovery of the (Ca + Mg)- and (Ca-CaM)-ATPase activities from the second sucrose gradient in the current study may, in part, be responsible for these discrepancies.

In assaying the ATPase activities of the membranes collected during the preparation of P_3B_2, we observed a significant difference between the synaptic plasma membrane and the microsomal membrane fractions. In our previous study of the endogenous ATPase activities of synaptic membranes (Sorensen and Mahler, 1981), we found that CaM could not significantly enhance (Ca + Mg)-ATPase activity unless the CaM still bound to these membranes

Table VIII. Distribution of ATPases in P_3B_2[a]

			ATPase				
	Protein	SDH[c]	Mg	Ca_H	Ca + Mg	Ca-CaM	Na + K
P_3[b]	165.9	9.92	260	215	26	53	290
P_3A[b], 0.32/0.51	0.61	ND	106	101	−4[d]	−4[d]	ND
P_3B[b], 0.51/1.1	95.0	3.04	272	280	65	91	348
P_3C[b] pellet	43.0	11.04	189	153	24	25	118
	(84)		(79)	(93)	(167)	(111)	
P_3B	91.1	3.04	272	280	65	91	348
P_3B_1, 0.32/0.77	6.3	0	337	372	1	13	562
P_3B_2, 0.77/0.91	14.3	1.44	300	338	42	66	480
P_3B_3, pellet	45.5	6.56	185	175	20	27	138
	(73)		(60)	(59)	(26)	(27)	

[a] P_3B_2 was prepared exactly as described by DeBlas and Mahler (1978). After collecting the various indicated interfaces from the sucrose density gradients, the material was diluted with cold distilled H_2O and centrifuged at 78,000 × *g* for 30 min and suspended in distilled H_2O. Protein and ATPase assays were as described in Experimental. SDH and Na + K-ATPase values were taken from DeBlas and Mahler (1978). Protein is expressed in mg/ml; ATPase specific activities, in $mU \times mg^{-1}$.

[b] Nomenclature of DeBlas and Mahler (1978).

[c] Values from DeBlas and Mahler (1978).

[d] Values below base line (Mg-ATPase).

had been removed by EGTA treatment (Sobue *et al.*, 1979; Grab *et al.*, 1979; Sorensen and Mahler, 1981). In contrast, the present experiments on the P_3B_2 preparation indicated that EGTA extraction of these microsomal membranes was not mandatory to show an effect by CaM on their (Ca + Mg)-ATPase activity. To determine whether this CaM stimulation indicated an absence of the modulator in these membranes, we extracted several of the microsomal fractions during the preparation of P_3B_2 with EGTA and assayed the resulting membranes for ATPase activity (Table IX). This treatment had little effect on the Mg- and Ca_H-ATPase activities present in the P_3, P_3B_2, and P_3B_3 fractions, while it led to a significant stimulation of the specific activities of both their (Ca + Mg)- and the (Ca-CaM)-ATPases without, however, affecting the extent of enhancement by CaM.

Discussion

As outlined in the Introduction, many neuronal functions are dependent on a change in the ionic permeability of the plasma membrane. Depolarization of the nerve terminal, mediated by a change in the flux of Na^+ and K^+ ions across the membrane, is responsible for increases in the intraterminal concentrations of Ca^{2+}, which serves as a trigger to activate several neuronal processes, including the mechanism for neurotransmitter release. Later the

Table IX. Distribution of ATPases in P_3B_2, Effect of EGTA Extraction[a]

		ATPase							
		Mg		Ca_H		Ca + Mg		Ca-CaM	
Fraction[b]	Extraction	SA (mU × mg^{-1})	% Change	SA (mU × mg^{-1})	% Change	SA (mU × mg^{-1})	% Change	SA (mU × mg^{-1})	% Change
P_3	No	260 ± 10	(100)	215 ± 8	(100)	26 ± 15	(100)	53 ± 22	(100)
	Yes	245 ± 7	94	175 ± 15	91	87 ± 18	335	132 ± 13	249
P_3B_2	No	300 ± 6	(100)	338 ± 17	(100)	42 ± 27	(100)	66 ± 40	(100)
	Yes	296 ± 18	99	303 ± 40	90	80 ± 9	190	133 ± 17	202
P_3B_3	No	185 ± 10	(100)	175 ± 1	(100)	20 ± 17	(100)	27 ± 20	(100)
	Yes	243 ± 27	131	186 ± 37	106	57 ± 34	285	90 ± 25	333

[a]The membrane fractions, P_3, P_3B_2, and P_3B_3, were prepared as described by DeBlas and Mahler (1978). These fractions were subjected to EGTA-extraction as previously described (Sorensen and Mahler, 1981) where indicated and then reexamined for the ATPase activities present. n = 3.

[b]Nomenclature of DeBlas and Mahler (1978).

ionic composition of the nerve terminal must be returned to its resting steady-state condition. ATPase activities of the plasma membrane are, in part, responsible for controlling the ionic composition of the nerve terminal by driving ion transport ions against an electrochemical gradient across the membrane (Barritt, 1981). In a previous report (Sorensen and Mahler, 1981), we described several ATPase activities found in a preparation of synaptic membranes from rat brain. In the present study we have assigned an intrasynaptosomal location for these activities through the measurement of the ATPase activities present in several well-defined subsynaptosomal fractions prepared from the same source.

In the first set of experiments, we compared the ATPase activities of intact synaptosomes with the activities measured in synaptosomes which had been disrupted by lysis or saponin treatment. We found that all ATPase activities were largely confined to the membrane fraction and absent from synaptoplasmic material (Table II). Although ATPase activities have been described for several of the internal components of the nerve terminal, including the cytoskeletal proteins myosin (Puszkin and Kochwa, 1974; Berl, 1975) or tubulin (White *et al.*, 1980) and the synaptic vesicles (Rothlein and Parsons, 1979; Michaelson *et al.*, 1980; Toll and Howard, 1980; Apps *et al.*, 1980), the results would indicate that, with the possible exception of the DA-sensitive ATPase, these activities contribute little to the complement of the ATPase activities of this particular synaptic membrane preparation. By itself this experiment does not rule out the possibility that some of the ATPase activity, especially the base line Mg^{2+}-ATPase, is due to the mitochondria in this preparation.

In the second set of experiments we compared enzyme activities in synaptosomes that were essentially intact to those that had sustained varying amounts of membrane disruption through saponin treatment or lysis. As suggested by Weller (1977), this type of experiment should—provided the synaptosomes are impermeable to various nucleoside phosphates—permit a distinction between ATP hydrolyzing sites localized on the interior aspect of the presynaptic membrane and those found on its exterior (junctional) aspect, as well as the postsynaptic membrane and its attachments. The former can effect the hydrolysis of ATP only after disruption of the synaptosomal membrane, while substantial ATPase activity found in intact synaptosomes would indicate a junctional localization, either in postsynaptic structures or, perhaps, of presynaptic ecto-ATPases. The results show that disruption of the synaptosome membrane resulted in a 4–5-fold stimulation of LDH activity over that measured in the intact synaptosomes. Of the ATPase activities assayed, the Mg and Ca_H-ATPase activities were not affected by disruption of the synaptosomal membrane, but the DA-, (Ca + Mg)-, and (Na + K)-ATPase activities were stimulated from 4- to 10-fold by this treatment.

Therefore, we conclude that the DA-, (Ca + Mg)- (with or without CaM), and (Na + K)-ATPases are located in the presynaptic membrane with their ATP hydrolysis sites largely facing the synaptoplasm. A similar location had been demonstrated for the ATP hydrolysis site of the (Na + K)-ATPase in the erythrocyte membrane (discussed in Stekhoven and Bonting, 1981). In the case of (Ca + Mg)-ATPases, the hydrolytic site appears to be located on the side from which Ca^{2+} is removed, which is constituted by the exterior aspect of the sarcoplasmic reticulum (Hasselbach, 1974) but the interior of the erythrocyte (Schatzmann and Bürgin, 1978; Sarkadi, 1980).

Several explanations can be entertained to explain the results with the Mg- and Ca_H-ATPases. These enzymes may be located: (a) in the presynaptic membrane with their ATP hydrolysis sites facing the synaptic junction; (b) on the postsynaptic membrane or its attachments; or (c) in the mitochondria. These possibilities were resolved in the next series of experiments.

We prepared several subsynaptosomal membrane fractions and assayed each for endogenous ATPase activity. We used the mitochondrial marker enzyme SDH to study the distribution of mitochondria in each of the membrane preparations. Because the (Na + K)-ATPase has been well characterized as a plasma membrane marker enzyme (discussed in Stekhoven and Bonting, 1981), we used this activity not only to establish its location at the nerve terminal, but also as an indicator of the presence of plasma membranes in each preparation. We chose four different membrane preparations which have been reported to vary in their content of different nerve terminal structures: (i) a synaptic membrane preparation that is largely devoid of attached postsynaptic structures (Table V); (ii) a synaptic membrane preparation containing attached postsynaptic structures (Table VI); (iii) a membrane preparation consisting largely of postsynaptic membranes with attached postsynaptic densities (Table VII); and (iv) a microsomal membrane preparation which is enriched in receptor function (Table VIII). In each of these preparations, the maximal activities for all of the ATPases examined (with apparent exceptions largely due to the low recoveries of certain activities) were found in the same fractions as the (Na + K)-ATPase. SDH activity was always localized in heavier fractions. These observations indicate that the majority of the ATPase activities being measured, including the Mg-ATPase, are intrinsic to plasma membranes and are not due to contaminating mitochondria. This inference is corroborated by previous experiments showing insensitivity to specific mitochondrial inhibitors, such as oligomycin and efrapeptin (Sorensen and Mahler, 1981).

In agreement with the data on intact synaptosomes, which indicated that the DA-, (Ca + Mg)-, and (Na + K)-ATPases were largely presynaptic, the results with the PSM preparation (Table VII) showed that these ATPase activities, as well as the Mg- and Ca_H-ATPase activities, are associated with

the lighter fractions from the sucrose gradient and not with PSM. These lighter fractions consist largely of presynaptic, microsomal, and junctional membranes. Also, the similarity of the levels of ATPase activities measured in synaptic membrane preparations either containing, or largely devoid of, postsynaptic attachments (compare Table V and Tables VI and VII) suggest that the presence of the postsynaptic membrane and its attached specializations contributes little to the ATPase activities measured, which, thus, are largely presynaptic. Finally, even though P_3B_2 consists of membranes which are enriched in receptor function, their high ATPase activities are largely presynaptic in origin. Their unusual properties (Table IX) are in agreement with the suggestion that they may consist of intracellular somatic membranes containing newly synthesized receptors (Roth *et al.*, 1982).

Thus, we can summarize the results obtained on the ATPase activities as follows: (1) all ATPases are largely contained in the presynaptic membrane; (2) the DA-, (Ca + Mg)- (and Ca-CaM-), and (Na + K)-ATPase are oriented with their ATP hydrolysis sites facing the synaptoplasm; (3) the Mg- and Ca_H-ATPases are oriented with their ATP hydrolysis sites on the junctional side of the presynaptic membrane and are therefore classified as ecto-ATPases of as yet unknown function.

Acknowledgments

This research was supported by a research grant NS 08309 from the National Institute of Neurological and Communicative Disorders and Stroke. H.R.M. is a recipient of a Research Career Award KO6 05060 from the Institute of General Medical Sciences.

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