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Review

The bioenergetics of neurotransmitter release

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Abbreviations: K_T, concentration required for half maximal transport; LDCV, large dense cored vesicle; SSV, small synaptic vesicle; VSCC, voltage-sensitive Ca^{2+}-channel; V_p, membrane potential across the plasma membrane; V_v, membrane potential across the synaptic vesicle membrane; V_{Na}, V_K, V_{Cl}, equilibrium potentials for Na^+, K^+, Cl^-.

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I. Introduction

The purpose of this article is to review the 'bioenergetic' approach to the nerve terminal and exocytosis, by which we mean the application of techniques similar to those used for investigating organelle and cellular bioenergetics, and to demonstrate that the synaptosome preparation can provide much more information than a simple demonstration of depolarization-induced transmitter release. To keep the length of the review within reasonable bounds it has been necessary to be selective. Since several recent reviews deal with the nature of terminal-specific proteins [1–7] and since the relationship between the structure and function of these proteins is only just beginning to be elucidated only superficial reference will be made to the proteins associated with exocytosis.

The nerve terminal responds to an action potential by exocytosing the contents of one or more synaptic vesicles into the synaptic cleft which separates the terminal from the post-synaptic membrane and contains receptors which are selective for the neurotransmitter. Ionotropic receptors possess an intrinsic ion channel, while metabotropic receptors are coupled, usually via G-proteins, to either a second messenger system or directly to an ion channel. The axon, which can vary in length from a few mm to 1 m, effectively isolates the terminal metabolism from that of the cell body. As a result the terminal can function as a bioenergetically autonomous 'cell', apart from specific axonal transport mechanisms allowing turnover of the protein and membrane components of the terminal. Indeed, it is possible, by controlled homogenization of brain tissue, to shear the axon just prior to the terminal and to produce isolated nerve terminals, or synaptosomes (e.g., Refs. 8–10), which are capable of maintaining a viable energy production for several hours. Synaptosomes contain the machinery for transducing an action potential into an influx of Ca^{2+} which induces synaptic vesicle exocytosis. At the same time they possess a wealth of highly active ion channels and carriers in their plasma membranes and are the targets for highly specific presynaptic neurotoxins. It is not surprising therefore that the synaptosome preparation has been widely exploited over the past 20 years.

The small size of most nerve terminals (about 1 μm in diameter) has virtually precluded the use of the direct electrophysiological techniques which have been so successful when applied to the post-synaptic events occurring on cell bodies (about 10 μm in diameter). This, together with the inherent complexity of the presynaptic release process, has meant that our knowledge of presynaptic events has lagged behind that of the post-synapse, where molecular biology and electrophysiology have supplied detailed information on the structure and function of the receptors responsible for controlling the electrical activity of the post-synaptic cell. Nevertheless, a bioenergetic approach to the nerve terminal and its synaptic vesicles has made a significant contribution to our understanding of the presynaptic mechanisms of uptake and storage of neurotransmitters and is beginning to provide some insight into that most elusive event – the molecular mechanism of fast-acting neurotransmitter exocytosis.

I-A. Transmitter classification

A convenient classification of neurotransmitters groups them into (i), amino acids, (ii) other 'classical' transmitters and (iii) neuropeptides [11]. The amino acid (Type 1) transmitters are the most abundant, being present in μmol/g mammalian brain, the major members of the class being glutamate, γ-aminobutyrate (GABA) and glycine. The widespread distribution of the amino acid transmitters in multiple neuronal and glial compartments, together with their involvement in general metabolic processes, means that their transmitter role is surprisingly difficult to study. However, accumulation into presynaptic vesicles, release on stimulation in the presence of Ca^{2+} and activation of postsynaptic receptors have all combined to confirm the transmitter role of these substances (for reviews see Refs. 12–15). GABA and glycine act on predominantly inhibitory receptors while glutamate is the main

excitatory transmitter. Generally, amino acid transmitters are 'fast acting', which reflects the speed both of presynaptic release and postsynaptic action.

Type 2 transmitters include the catecholamines (dopamine (DA), adrenaline and noradrenaline (NA)), 5-hydroxytryptamine (5-HT), purines and acetylcholine (ACh), which are all present at nmol/g mammalian brain. Their uptake and storage mechanisms are closely similar to those for the amino acids, although detailed differences in release mechanisms exist: in particular, the probability of vesicle release when an action potential reaches a catecholamine release site is much lower than for ACh or the amino acids [16].

Neuropeptides (classified as Type 3 transmitters) are co-transmitters at many terminals [17–21] and are present at concentrations ranging from nmol to pmol/g brain. They are stored in large dense cored vesicles (LDCVs), and are released from a variety of sites on the neurone and so differ from the small synaptic vesicles (SSVs) containing Type 1 and some of the Type 2 transmitters which are generally released at synapses directly onto the postsynaptic receptors [22–24]. It is necessary to emphasize that the mechanisms by which SSVs and LDCVs are released differs in several fundamental aspects (Section VIII) such that there is no universal exocytotic mechanism.

II. The synaptosomal preparation

Mammalian synaptosomes can be most effectively made from brain regions such as the cerebral cortex and the hippocampus which have a clearly defined layered structure. A crude mitochondrial pellet containing predominantly a mixture of synaptosomes and free mitochondria is subjected to either density gradient centrifugation in a Ficoll gradient (e.g., Ref. 25), or rate sedimentation in a Percoll gradient (e.g., Ref. 9).

The synaptosomal preparation has particular advantages and problems. It has undeservedly gained a somewhat dubious reputation among neuroscientists because many early experiments were carried out under conditions which were difficult to relate to the physiological events of neurotransmission and because the preparation was considered to be in some way 'damaged'. However, as we shall now discuss, carefully prepared Ficoll- or Percoll-purified synaptosomes are predominantly intact, show high respiratory control [26,27], maintain a plasma membrane potential of -60 to -80 mV in low K^+ medium [28–33] and a cytoplasmic free Ca^{2+} concentration of 0.1–0.2 μM [30,34–41] and release neurotransmitter by mechanisms which respond to the same inhibitors and modulators as more complex preparations (see Section VIII).

II-A. The problem of transmitter heterogeneity

Mammalian synaptosomes are inherently heterogeneous in terms of transmitter content, since even the most closely defined anatomical regions contain a wide variety of synapses. Various immunological techniques have been used to endeavour to purify synaptosomal subpopulations from mammalian brain; the most successful has exploited a surface antigen from the synaptosomal plasma membrane of the purely cholinergic electric organ of the ray, *Torpedo marmorata* [42,43]. The antigenic determinant, termed *Chol I*, is actually a family of minor gangliosides which are located specifically on the plasma membrane of cholinergic terminals from mammalian or non-mammalian sources. An 18-fold purification of cholinergic synaptosomes from rat cerebral cortex can be obtained by exploiting anti-*Chol I* immunobeads [42,44]. The same group [45] has also purified glutamatergic and GABAergic synaptosomes from the neuromuscular junction of Dublin Bay prawns.

What are the uses and limitations of a synaptosome preparation with a heterogeneous transmitter content? No evidence exists to indicate that nerve terminals from a given brain region are heterogeneous with respect to their basic bioenergetic functions, i.e., glycolysis and oxidative phosphorylation and the maintenance of plasma membrane ion gradients and membrane potentials. Thus, the uptake or release of a particular transmitter, when these bioenergetic parameters are uniformly altered in the population, can be reliably studied. Synaptosomal heterogeneity does create problems when only a subpopulation possesses a specific control mechanism, for example a presynaptic receptor, since the effect that this pathway might have on any second messenger, ion flux or protein phosphorylation mechanism in the population would be diluted by the unresponsive synaptosomes.

One way to minimise the problems of transmitter heterogeneity is to select the transmitter which is released in greatest abundance from a given brain area. In most cases this is glutamate, which is by far the dominant excitatory amino acid in the mammalian central nervous system [12,46]. Together with GABA (or glycine for the spinal cord), the two amino acids probably account for 90% of all synaptic transmission in areas such as the cerebral cortex. Glutamate is of additional interest, since it is implicated in theories of memory and learning [47–49], while the uncontrolled release of glutamate when the brain is deprived of oxygen underlies much of the irreversible damage occurring during stroke [50–53].

III. Synaptosomal energetics

Within the small (0.7–1 μm) diameter of the terminal there are a mitochondrion, several small synaptic vesicles (SSVs) of diameter approx. 50 nm together with the occasional large dense cored vesicle (LDCV). There is little internal membrane corresponding to endoplasmic reticulum [54–56].

Nerve terminals are bioenergetically autonomous and synaptosomes continue to function for at least 6 h after preparation when supplied with glucose. Inhibition of oxidative phosphorylation results in a 10-fold increase in the rate of glycolysis [26,57]. The magnitude of this 'Pasteur effect' demonstrates that essentially all the synaptosomes contain functional mitochondria, whose ATP synthesis is normally restricted by glycolysis. However, despite this large glycolytic capacity there is a significant fall in overall ATP/ADP ratio within the synaptosomes when oxidative phosphorylation is blocked [26,57]. For reasons which are not clearly understood, synaptosomal glycolysis fails after a few minutes of anoxia or inhibition of cytochrome oxidase, conditions under which the respiratory chain becomes very reduced, but is maintained indefinitely after rotenone, when the respiratory chain components remain oxidized [57].

Synaptosomes have negligible endogenous glucose or glycogen stores and can readily be made hypoglycaemic by removal of glucose from the medium [58]. The only other substrate that can be utilized by intact synaptosomes is pyruvate [58]. The high concentrations of glutamate and aspartate in the synaptosomes cannot be utilized for oxidative metabolism [27].

The intrasynaptosomal mitochondria utilize only about 10% of their respiratory capacity to maintain ATP levels in the resting terminal, since addition of protonophore causes a large stimulation of respiration [26]. The major ATP requirement in the terminal is for the (Na^+/K^+)-ATPase, but ATP is also necessary for Ca^{2+} homeostasis, maintaining a proton electrochemical gradient across the synaptic vesicle membrane and also for some undefined aspect of the exocytotic process itself (Fig. 1).

III-A. Na^+ and K^+-circuits across the synaptosomal plasma membrane

In vitro, synaptosomes retain the physiological state of the neuronal plasma membrane with a high resting conductance to K^+ and a low Na^+ conductance [59,60]

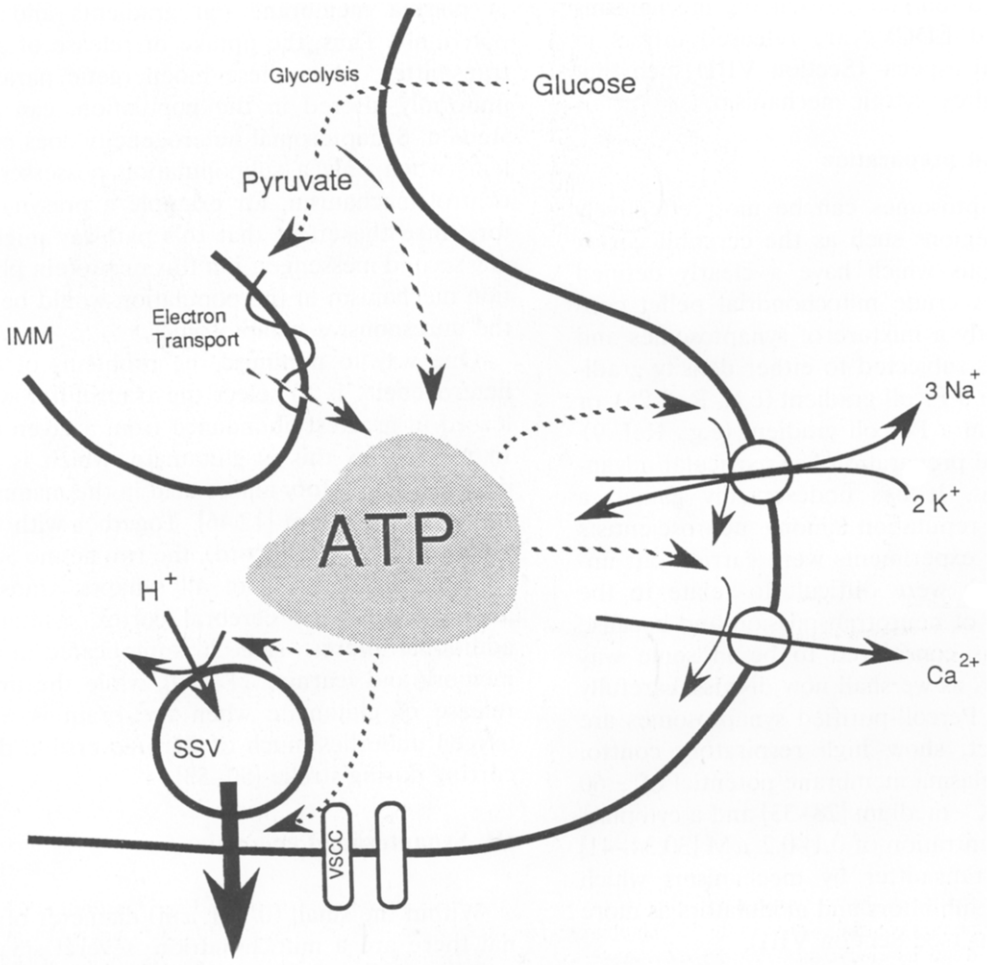

Fig. 1. Major pathways for the synthesis and utilization of ATP in synaptosomes. Isolated nerve terminals can utilize both glucose and pyruvate. The major ATP utilising process is the Na^+/K^+-ATPase, particularly if the Na^+ conductance of the membrane is increased, when dissipative Na^+ cycling can utilize most of the mitocvhondrial respiratory capacity. ATP is also required for the plasma membrane Ca^{2+}-ATPase and for an as yet unidentified component of the exocytotic process in addition to the maintenance of the V-ATPase.

(Fig. 2). As a result the plasma membrane potential, V_p, is approximated by the equilibrium potential for the K^+ gradient (or the more convenient $^{86}Rb^+$) across the membrane:

$$V_p = 2.3RT/F(\log_{10}\{[K^+]_o/[K^+]_i\}) \quad (1)$$

By this technique it is found that synaptosomes respiring in 3–5 mM K^+-containing media maintain a plasma membrane potential in the region of −70 mV [28,29,31]. The parallel use of a lipophilic cation such as tetraphenylphosphonium, which is further accumulated into the mitochondrion, is consistent with the expected mitochondrial membrane potential of about −150 mV [29]. The very low resting Na^+ conductance of the terminal membrane is apparent when the (Na^+/K^+)-ATPase is inhibited by ouabain: the plasma membrane depolarizes at a rate of only 1 mV/min [57].

The large K^+ conductance of the plasma membrane must mean that there is a subclass of K^+-channels that remain open in the resting terminal; however, these have not been identified. An important channel for stabilizing the resting potential of synaptosomes is the K_A-channel, which is closed at normal resting potentials but rapidly activates when the membrane becomes slightly depolarized, thus restoring the potential [61]. inhibition of this channel by 4-aminopyridine, 4AP [30,62] or dendrotoxin [62–64] can initiate spontaneous

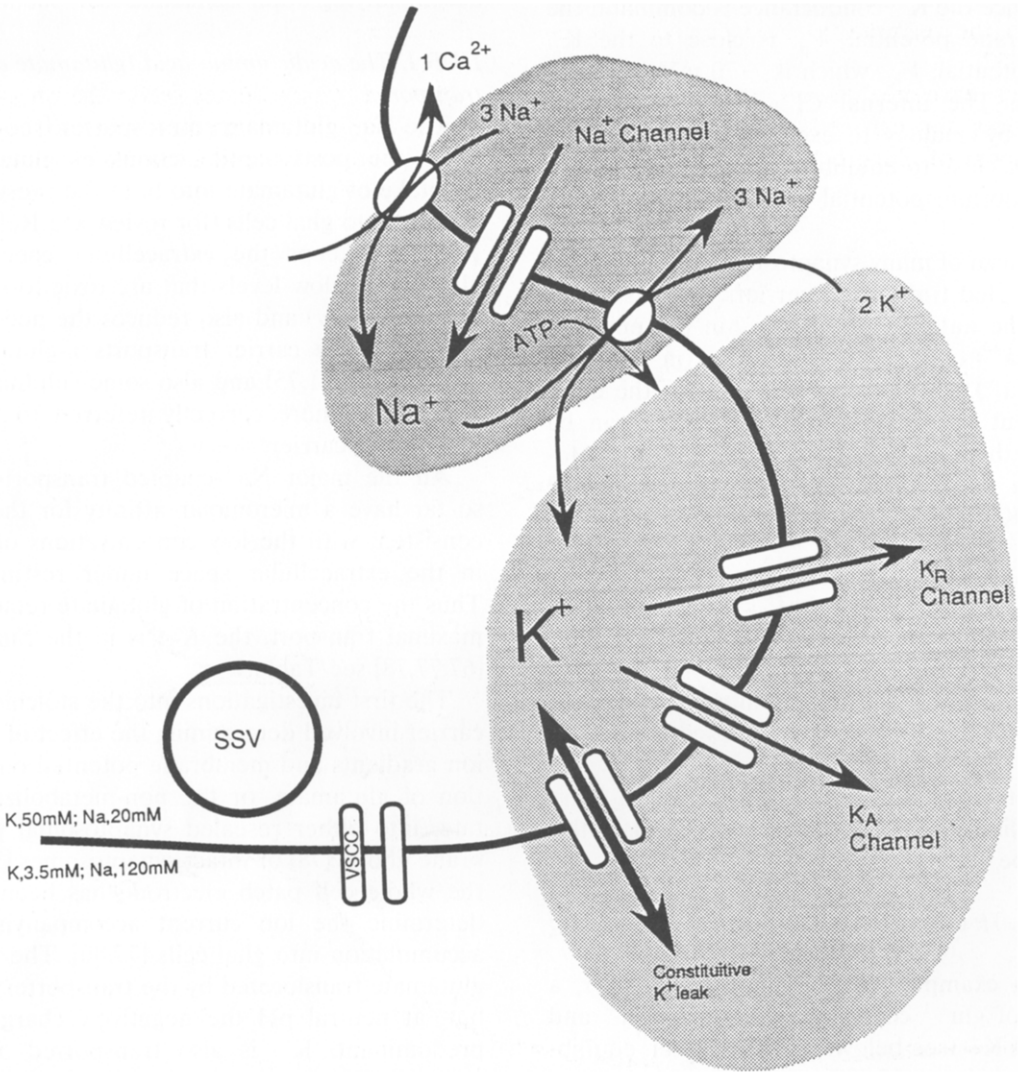

Fig. 2. Na^+ and K+ circuits across the synaptosomal plasma membrane. The polarized terminal possesses a large constitutive K^+ conductance such that the plasma membrane potential is close to the K^+ equilibrium potential (about −70 mV). The Na^+ equilibrium potential is about +50 mV, and Na^+ transport down its electrochemical gradient can be coupled to the expulsion of Ca^{2+} via the Na^+/Ca^{2+} exchanger and the accumulation of neurotransmitters and precursors by Na^+-coupled co-transport (not shown). The plasma membrane may be depolarized by increasing $[K^+]_{out}$, by activating the voltage-activated Na^+ channels with veratridine, or by inhibiting the K_A channel with 4-aminopyridine or dendrotoxin. In this last case the synaptosomes fire repetitive action potentials involving both the Na^+ channels and the delayed rectifier K^+ channel, K_R.

action potentials in synaptosomes (see subsection VI-C).

III-B. The thermodynamics of Na^+-coupled transmitter carriers

In physiological media containing 120 mM Na^+, 120 mM Cl^- and 3.5 mM K^+, synaptosomes maintain an internal [Na^+] of 20 mM and an internal [K^+] of about 50 mM (this low value being due to the transient loss ions during the shearing of terminals from their axons, the loss being compensated by sucrose entry) [29,65]. The Na^+ equilibrium potential, V_{Na} (the value of the membrane potential at which the Na^+ gradient would be at equilibrium), is +47 mV (see the analogous Eqn. 1), although since the K^+ conductance is dominant, the plasma membrane potential, V_p, is close to the K^+ equilibrium potential, V_K, which is −70 mV (see subsection III-A). The internal Cl^- is not known for certainty, but by analogy to excitable cells would be predicted to be close to equilibrium, i.e., about 8 mM; thus, the equilibrium potential for Cl^- would also be −70 mV.

The mechanism of many synaptosomal plasma membrane Na^+-coupled transmitter carriers involves in addition either the antiport of K^+ (i.e., in the opposite direction to Na^+) or the symport of Cl^- (in the same direction as Na^+). The general equation for the equilibrium gradient of a transmitter 'Tr' which can be transported with t positive charges, i.e., as Tr^{t+} together with n Na^+ ions and c Cl^- ions and the antiport of k K^+ ions is given (at 37 °C) by:

$$\log_{10}\{[Tr]_{in}/[Tr]_{out}\} = \{nV_{Na} - cV_{Cl} - kV_K - (t + n - c - k)V_p\}/61.52 \quad (2)$$

where V_{Na}, V_{Cl} and V_K are the equilibrium potentials (in millivolts) for Na^+, Cl^- and K^+, respectively, and the numerical factor is $2.3RT/F$ (again in mV) at 37 °C. Since under resting conditions Cl^- and K^+ are close to equilibrium, such that $V_{Cl} = V_K = V_p$, this equation can be simplified to:

$$\log_{10}\{[Tr]_{in}/[Tr]_{out}\} = \{nV_{Na} - (t + n)V_p\}61.52 \quad (3)$$

To take as an example the glutamate transporter, a stoichiometry of glu^{-1} cotransported with 3 Na^+ and in antiport to 1 K^+ (see below) would allow an equilibrium gradient of almost 37000 to be maintained across the plasma membrane of a polarized synaptosome, based on the experimentally determined gradients discussed above.

An important consequence of Eqns. 2 and 3 is that if the carrier is electrogenic (i.e., there is net charge movement across the membrane) then the equilibrium distribution of the transmitter is responsive both to the plasma membrane potential and to the Na^+ concentration gradient. In the case of the amino acid neurotransmitters, glutamate, GABA and glycine, whose plasma membrane carriers are close to equilibrium [66–68], there is a high concentration of the amino acid within the terminal cytoplasm. A sustained decrease in membrane potential, Na^+ gradient or both parameters will lead therefore to an efflux of the transmitter across the plasma membrane by reversal of the carrier [68]. it should be emphasized that this is not a normal mode of transmitter release, since physiological depolarization is so transient, but it is a factor complicating in vitro experimentation (see Section 6) and is of profound significance during the prolonged energy deprivation accompanying brain ischaemia (see Section IX).

III-B.1. The acidic amino-acid (glutamate and aspartate) transporter

The Na^+-glutamate cotransporter (see Fig. 3) terminates the postsynaptic actions of glutamate by the reuptake of glutamate into both the presynaptic terminal and into glial cells (for review see Refs. 67, 69–71). Reuptake keeps the extracellular concentrations of glutamate below levels that are toxic to neurones [72–74] (see below) and also reduces the need for de novo synthesis. The carrier transports L-glutamate, L- and D-aspartate [71,75] and also some sulphur amino-acids [76] and is more correctly referred to as the acidic amino-acid carrier.

All the major Na^+-coupled transporters examined so far have a micromolar affinity for their substrates consistent with the low concentrations of transmitters in the extracellular space under resting conditions. Thus the concentration of glutamate required for half-maximal transport, the K_T, is in the range 2–50 μM [67,77,78] see Table I.

The first investigations into the stoichiometry of the carrier involved determining the effect of alterations in ion gradients and membrane potential on the distribution of glutamate, or the non-metabolizable D-aspartate, into either resealed synaptosomal plasma membrane ghosts [78] or intact synaptosomes [79]. Recently, the whole cell patch electrode has been exploited to determine the ion current accompanying glutamate accumulation into glial cells [77,80]. The ionic form of glutamate translocated by the transporter is not known but, at neutral pH the negatively charged species is predominant. K^+ is also transported and the stoichiometry of the carrier is now postulated to be the cotransport of a glutamate anion with 3 Na^+ (or 2 Na^+ and 1 H^+) in exchange for 1 K^+ [77]. From Eqn. 3, the former stoichiometry would allow a 35000-fold gradient to be maintained between the extracellular fluid and the cytoplasm of glutamatergic neurones. Because neither the exact proportion of glutamatergic synapto-

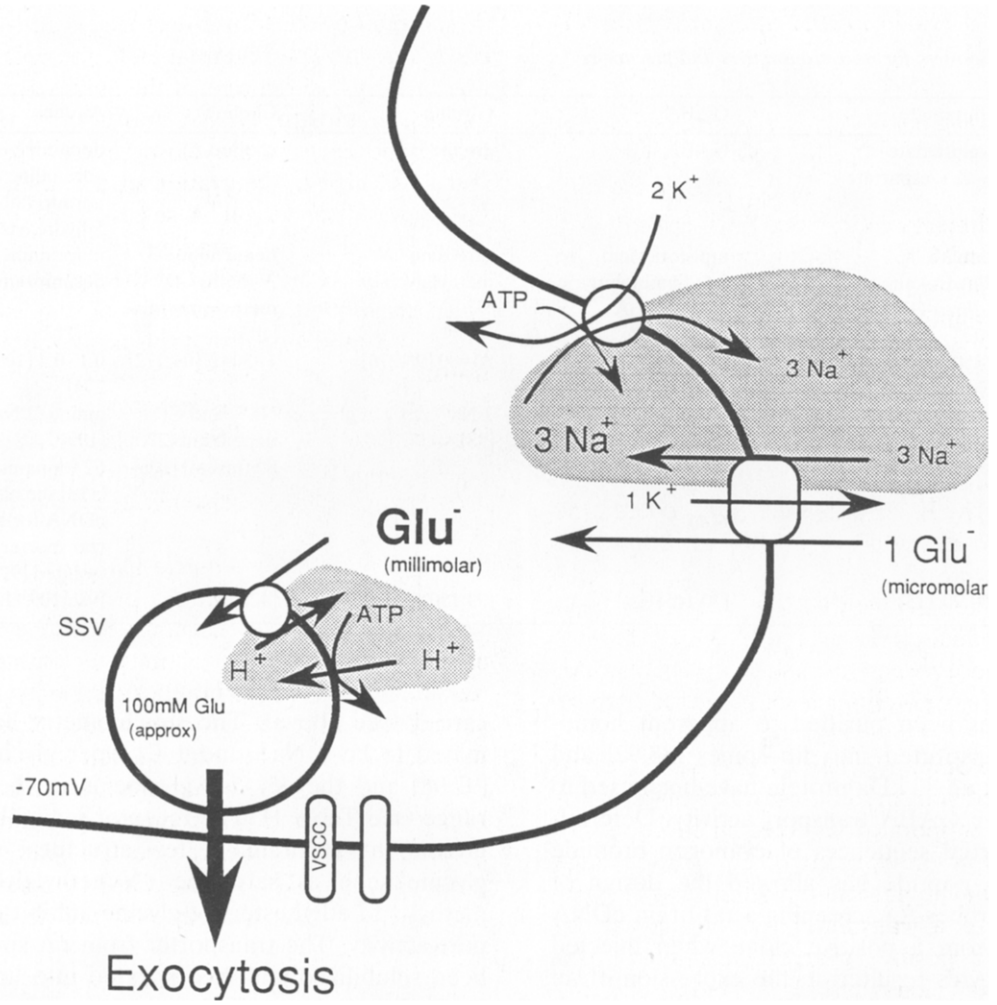

Fig. 3. Glutamate compartments in the glutamatergic nerve terminal. Glutamate is accumulated across the plasma membrane by cotransport with Na^+ (and counter transport of K^+). The carrier is capable of maintaining a millimolar cytoplasmic glutamate with an extraterminal glutamate concentration in the micromolar region. Cytoplasmic glutamate can be released directly under in vitro and pathological conditions of chronic depolarization by thermodynamic reversal of the glutamate carrier. The transvesicular carrier accumulated the glutamate anion driven by the positive interior membrane potential maintained by the V-ATPase. An approx. 10-fold gradient of glutamate is maintained across the vesicular membrane. Cytoplasmic aspartate can be released by reversal of the plasma membrane carrier, but little or no accumulation of the amino-acid occurs into the vesicles.

somes in a preparation nor the quantitative distribution of glutamate within the terminal is known, it is not possible to be precise about the cytoplasmic glutamate concentration; however, it is likely to be in the region of 1–10 mM, implying a gradient of 1000–10000 across the plasma membrane, i.e., within the thermodynamic capabilities of the carrier.

The acidic amino-acid transporter from rat brain has been purified to near homogeneity [81]. The main polypeptide purified is 80 kDa and on reconstitution retains its ionic dependence, electrogenicity, affinity, substrate specificity and stereospecificity.

III-B.2. The GABA transporters

As in the case of glutamate, GABA is retrieved into the terminal by a high-affinity Na^+-coupled carrier with a K_T in the micromolar range [82,83]. Immunocytochemistry reveals that GABA [84,85] and the GABA carrier [86] are concentrated in the terminals of GABAergic neurones, although a closely related carrier is present in glia. The two carriers can be distinguished by their inhibitor specificities: the terminal carrier can be competitively inhibited by the transportable analogue 2,4-diaminobutyrate, while the glial carrier is sensitive to β-alanine [87].

The terminal transporter has been studied in detail by the group of Kanner [82,83,88–91] and they conclude that the probable stoichiometry of the carrier is a symport of 2 Na^+ and 1 Cl^- with the neutral GABA zwitterion [83] but with the possibilities of more complex stoichiometries not excluded. This stoichiometry would allow an equilibrium gradient of 6000 to be established across the plasma membrane of a resting synaptosome (see Eqns. 2 and 3).

TABLE I

Plasma membane transporters for neurotransmitters and precursors

	Glutamate	GABA	Glycine	Choline	Amines
Substrates	L-glutamate D- & L-aspartate	GABA	glycine	choline	dopamine adrenaline noradrenaline 5-hydroxytryptamine
Inhibitors	kainate dihydrokainate L-*threo*-OH-D-aspartate	nipecotic acid 2,4-diaminobutyric acid (DABA)	sarcosine (*N*-methylglycine)	hemicholine-3 *N*-methyl-4-methylpiperidine [103]	imipramine desimipramine
K_T (μM)	2–50 (glutamate)	2–4 [95]	30–80 [98,276]	1–3 [42,104,272]	0.1–0.4 [109,112, 274]
Stoichiometry	1 Glu^- :2Na^+ :1 H^+/1K^+ or 1 Glu^- :3 Na^+/1 K^+	2 Na^+ :1 Cl^- :1 GABA	2 Na^+ :1 Cl^- : glycine [98]	Na^+- and Cl^--dependent [273]	amine:2 Na^+ :Cl^-/2 K^+ [109]
Mass (kDa)	80 (rat brain)	80		80 (insect) [105]	62 (dopamine) [113] see also [71]
Gene		*GAT 1*			cDNA for human NA transporter has been isolated [92a]
General references	[69,70,115]	[70,115]	[99,150,276]	[42,102,105]	[92a,109,112,275]

The carrier has been purified to apparent homogeneity and reconstituted into liposomes [88,92] and antibodies against an 80 kDa protein have been used to immunoprecipitate GABA transport activity. Determination of the partial sequences of cyanogen bromide fragments of the peptide has allowed the design of oligonucleotide probes for screening a rat brain cDNA library. mRNA from a positive clone when injected into *Xenopus* oocytes resulted in the expression of an Na^+-dependent, 2,4-diaminobutyrate-sensitive [^{3}H]-GABA uptake pathway which bound polyclonal antibodies against the conventionally purified transporter [92]. A human brain GABA transporter has also been cloned and sequenced [93]. Both proteins have 599 amino-acids, a calculated molecular mass of 67-kDa and twelve potential transmembrane segments [92]. The recently cloned human noradrenaline transporter shows a striking overall amino-acid identity of 46% with the human GABA transporter and, allowing for conservative amino-acid substitutions, the similarity increases to 68% [92a]. Thus, by analogy to the receptor and channel superfamilies, the neuronal Na^+-cotransporters for different neurotransmitters may also belong to a gene family. Distinct high-affinity GABA transporters can be distinguished pharmacologically in isolated membrane vesicles and in liposomes with reconstituted carriers [94] and a low-affinity GABA transporter may also exist [95].

III-B.3. The glycine transporter

Glycine is an inhibitory transmitter in the spinal cord. The transporter has a high-affinity for glycine, is electrogenic and catalyses the cotransport of Na^+, Cl^- and glycine [96,97], i.e., it is similar to the GABA carrier (see above). The stoichiometry has been estimated to be 2 Na^+ and 1 Cl^- per glycine zwitterion [97,98] and the K_T for glycine is in the micromolar range (see Table I). The receptor is highly specific for glycine in rat brain. A few structural analogues of glycine such as sarcosine (*N*-methylglycine) or the methyl and ethyl esters of glycine, inhibit glycine transport activity. The transporter from rat spinal cord has been solubilized and incorporated into liposomes [99]. Both the glycine and the glutamate transporters are inhibited in a dose-dependent manner by arachidonic acid [100,101].

III-B.4. The choline transporter

Released acetylcholine is not retrieved into nerve terminals but is degraded by acetylcholinesterase to acetate and choline. The choline is retrieved by a Na^+-coupled cotransporter, which can be inhibited by hemicholinium-3 and related compounds [102–104]. Both a high-affinity and a low-affinity carrier have been reconstituted into proteoliposomes, and display an ion dependence and pharmacological profile identical to that of intact synaptosomes [105,106]. Cholinergic nerve terminals which have been affinity purified from rat cortical synaptosomes (using *Chol 1* antibody, see above) have a high-affinity choline transporter with K_T 2.7 μM and requiring Na^+ and Cl^- [42]. Uptake is reported to be inhibited by muscarinic agonists and enhanced by octopamine [107].

III-B.5. Biogenic amine transporters

5-Hydroxytryptamine (5-HT), dopamine (DA), adrenaline and noradrenaline (NA) are all transported by Na^+-coupled transporters which are sensitive to tri-

cyclic antidepressants (e.g., imipramine). The different amine transporters can be distinguished by their inhibitor sensitivities [108]. Desimipramine is a much weaker inhibitor of 5-HT transport than of NA in brain tissue, while DA transport is relatively insensitive to both imipramine and desimipramine but is sensitive to buproprion (see Ref. 71).

Study of the 5-HT transporter has been carried out mostly in platelets but has also been examined in mouse brain membrane vesicles where Na^+, Cl^- and 5-HT are cotransported in exchange for K^+. Transport is proposed to be electroneutral [109]. The NA transporter in rat heart adrenergic nerves depends on external chloride [110], as does NA accumulation into PC12 cells (a cell line derived from adrenal chromaffin cells) where internal K^+ is also required [111]. A high-affinity 5-HT transporter has been genetically reconstituted in a clonal cell line in which 5HT transport is normally absent [112]. The transporter was specific for 5-HT, was inhibited by imipramine and has a high-affinity for 5-HT (K_T 0.39 μM). A high-affinity photoaffinity label for the dopamine transporter has been incorporated into a striatal polypeptide of apparent M_r 62000 [113].

A cDNA clone has been isolated for the human noradrenaline transporter [92a] that has a high sequence similarilty with the previously cloned GABA transporter (see subsection III-B.2). The sequence predicts a protein of 617 amino-acids with 12–13 potential membrane-spanning regions, again similar to that predicted for the GABA transporter.

IV. Synaptic vesicles

There are two classes of synaptic vesicle in the brain (for reviews see Refs. 114–116). Small synaptic vesicles (SSVs) with a diameter of 50 nm are electron lucid, contain the 'classical' biogenic amine or amino-acid transmitters and undergo local recycling in the terminal without reference to the cell body [5,117,118]. Some SSVs containing catecholamines do not appear electron lucid but develop an artifactual electron-dense core in certain stains. Large dense-core vesicles (LDCVs) with a diameter > 70 nm, on the other hand, contain precursor and processed neuropeptides and must be recycled to the cell body for processing in the Golgi apparatus [5]. The exocytotic mechanisms for the two are distinct: LDCV release is not restricted to active zones in terminals [22,119,120], see Section VIII. A close parallel for the study of LDCV release is the chromaffin vesicle from adrenal medullary cells [121–123], but the extremely rapid exocytosis of SSVs is unique to the nervous system and cannot be extrapolated from studies on non-neuronal systems.

Synaptic vesicles possess a vacuolar V-type proton-translocating ATPase (for review see Refs. 114,124–127) which pumps protons into the lumen of the vesicle. The V-ATPase is insensitive to azide, vanadate, oligomycin and ouabain, but is sensitive to NEM and is also inhibited by bafilomycin A1, a *Streptomyces* antibiotic [128].

Except in the case of model systems such as the purely cholinergic SSVs from the *Torpedo* electric organ [114,129,130], it is not feasible to obtain a preparation of SSVs which is homogeneous with respect to neurotransmitter content. As in the case of synaptosomes, it must be born in mind that only a proportion of the preparation will contain or be capable of accumulating a given neurotransmitter. Furthermore, synaptic vesicles differ in the stability of their stored neurotransmitter. Thus, even after long centrifugation on sucrose density gradients it is still possible to obtain a vesicle fraction which contains much of the original biogenic amines (acetylcholine, NA, 5-HT etc.), while the same preparation is virtually devoid of amino acids [131,132]. The lability of the vesicular glutamate store contrasts with that for ACh and catecholamines (see below) which can maintain their stores in the absence of ATP due to intravesicular complexation.

This initial failure to locate amino acids within the synaptic vesicle fraction led to much early confusion as to whether amino-acids were released by an exocytotic mechanism (for discussion see Ref. 15). Amino-acid vesicles do not retain their contents in the absence of a proton electrochemical potential [133]. However, the availability of rapid purification techniques for synaptic vesicles [134–136] and the realization that the leakage of amino acids from vesicles can be prevented by including ATP in the preparation medium to prevent the collapse of the vesicular proton gradient together with high concentrations of *N*-ethylmaleimide to block the vesicular carriers [137], has allowed the isolation of vesicle preparations containing high concentrations of glutamate [137].

IV-A. Synaptic vesicle carriers

For all transmitters studied, accumulation into vesicles is driven by the transvesicular proton electrochemical gradient. However, the component used (vesicular membrane potential, V_v, or ΔpH) varies [114,115,135, 136,138–145]. As in other bioenergetic systems the relative contributions of the two components can be varied in vitro. The low electrical capacity of the synaptic vesicle membrane means that very little proton translocation can occur before a large membrane potential, positive inside, builds up. In the presence of an anion such as Cl^-, which is permeable across the vesicle membrane by electrical uniport, the membrane potential is diminished, allowing the ΔpH component to increase. Conversely, when K^+ is present on both side of the vesicle membrane at similar concentrations, nigericin can selectively collapse ΔpH.

These bioenergetic techniques were first exploited for the chromaffin vesicle [146,147] and platelet granule [146], although it must be born in mind that these are primarily models for the LDCV.

IV-A.1. The vesicular glutamate carrier

Although initial preparations of synaptic vesicles contained no significant amino acids [132], in the early 1980's it was found that synaptic vesicles which were partially [148] or more extensively [134] purified were capable of accumulating radio-labeled glutamate in an ATP-dependent manner [134,135,138,143,145].

The vesicular glutamate transporter can be readily distinguished from that at the plasma membrane (Fig. 3). Firstly, it is dependent on ATP and a proton-rather than a sodium-electrochemical potential gradient [134,138]. Secondly, it has a more stringent amino-acid specificity than the plasma membrane carrier. No amino-acid other than L-glutamate itself competes with labeled L-glutamate for uptake into the vesicle preparation [135,138]; in particular aspartate is ineffective [138] and there are no reports of L-aspartate uptake into a separate vesicle population. Since the plasma membrane acidic amino acid carrier does not distinguish between L-glutamate and L-aspartate, and since the two amino-acids are in any case interconvertible in the cytoplasm, it is the vesicular carrier which gives the glutamatergic specificity to the terminal.

A third distinction between the plasma membrane and vesicular carrier is the concentration of glutamate required for half-maximal transport (K_T). Reflecting the high concentrations of glutamate present in the cytoplasm, the vesicular carrier has a millimolar K_T for the amino-acid [115,138], rather than the micromolar K_T at the plasma membrane. The concentration gradient of glutamate across the vesicle membrane is probably less than 10-fold [115].

Experimental manipulation of the transvesicular membrane potential V_v and pH gradient shows that ATP-driven glutamate uptake is optimal when V_v is high and ΔpH low [135,145], consistent with the electrophoretic accumulation of the glutamate anion (Fig. 3). High [Cl^-] collapses V_v and inhibits uptake; however, some chloride is essential for the accumulation of glutamate and may exchange for glutamate, although this remains speculative [138,143,145]. Reconstitution of transporters into liposomes will more clearly define the carrier mechanism [149].

IV-A.2. The vesicular GABA and glycine carriers

ATP-dependent uptake of GABA [136,139,142] and glycine [142,150] into a synaptic vesicle fraction can be detected. However, due to the lower extent of uptake in the mixed population compared with glutamate, less is known about the transport mechanism. GABA uptake is dependent on both ΔpH and V_v [151] and in contrast to glutamate, neither GABA or glycine uptake into synaptic vesicles is greatly stimulated by low Cl^- concentrations [142]. Both vesicular carriers have a mM K_T (see Table II).

IV-A.3. The vesicular ACh carrier

Most information on the synaptic vesicle acetylcholine transporter has come from *Torpedo* electric organ [129,152–154]. Uptake is ATP-dependent and uncoupler-sensitive [155] with a K_T in the micromolar range [156]. Studies of the carrier have been aided by the existence of a specific inhibitor for the carrier, vesamicol, formerly called AH5183 [157–159]. The inhibitor does not block the H^+-translocating ATPase, nor dissipate the proton electrochemical gradient but binds non-competitively to a regulatory site distinct from the acetylcholine binding site [158] and present at a density of approx. 4 per vesicle [158]. Uptake of

TABLE 2

Synaptic vesicle transporters for neurotransmitters

	Glutamate	GABA	Glycine	Acetylcholine	Amine
Substrates	L-glutamate	GABA	glycine	acetylcholine	dopamine adrenaline noradrenaline 5-hydroxytryptamine
Inhibitors	D-glutamate bromocriptine [144]	none of the plasma membrane inhibitors works	GABA [150] β-alanine [150]	vesamicol (AH 5183)	reserpine Tetrabenazine
Cl^--stimulated	yes [150]	no [142]	very little [142,150]		
Reliance on V_p or ΔpH	$V_p \gg \Delta$pH [115,145]	$V_p \equiv \Delta$pH	?	$V_p \ll \Delta$pH	$V_p \ll \Delta$pH
K_T	0.2–1.6 mM [115,148, 279]	5–7 mM [139,142]	8–9 mM [142,150]	300 μM (*Torpedo*) [249]	0.1–4 μM [114,278–281]
General References	[115,138,145]	[135,139,142]	[142,150]	[114,159]	[70,114,282]

acetylcholine is dependent both on the pH gradient and the vesicle membrane potential, but the stoichiometry has not been established unequivocally [155].

IV-A.4. The vesicular catecholamine and 5-HT carriers

As a result of the availability of the secretory chromaffin and platelet vesicles, which can transport and store catecholamines and 5-HT, there is considerable information about these carriers (for review see Ref. 114). ATP-dependent catecholamine transport into chromaffin vesicles is inhibited by reserpine with a K_i of about 0.1 nM. Reserpine inhibits transport, but not the ATPase or the generation of the proton electrochemical gradient. Although these vesicles are primarily models of LDCVs, reserpine will also inhibit the CNS transporter. The carrier has a low specificity, since reserpine inhibits the uptake of adrenaline, DA, NA and 5-HT into isolated chromaffin and synaptic vesicles. However, transport is stereoselective [160].

The stoichiometry of the carrier has been recently reviewed [114]. Briefly, the ΔpH component of the proton electrochemical potential is essential for amine uptake. Thus addition of nigericin to discharge any pH gradient inhibits the ATP-driven uptake of adrenaline. A direct role of ΔpH can be shown in the absence of ATP by generating an artificial pH gradient which can drive adrenaline uptake. However, transport is also electrogenic, since a membrane potential, positive inside, generated by suspending K^+-free vesicles in KCl and adding valinomycin also drives uptake. Taking

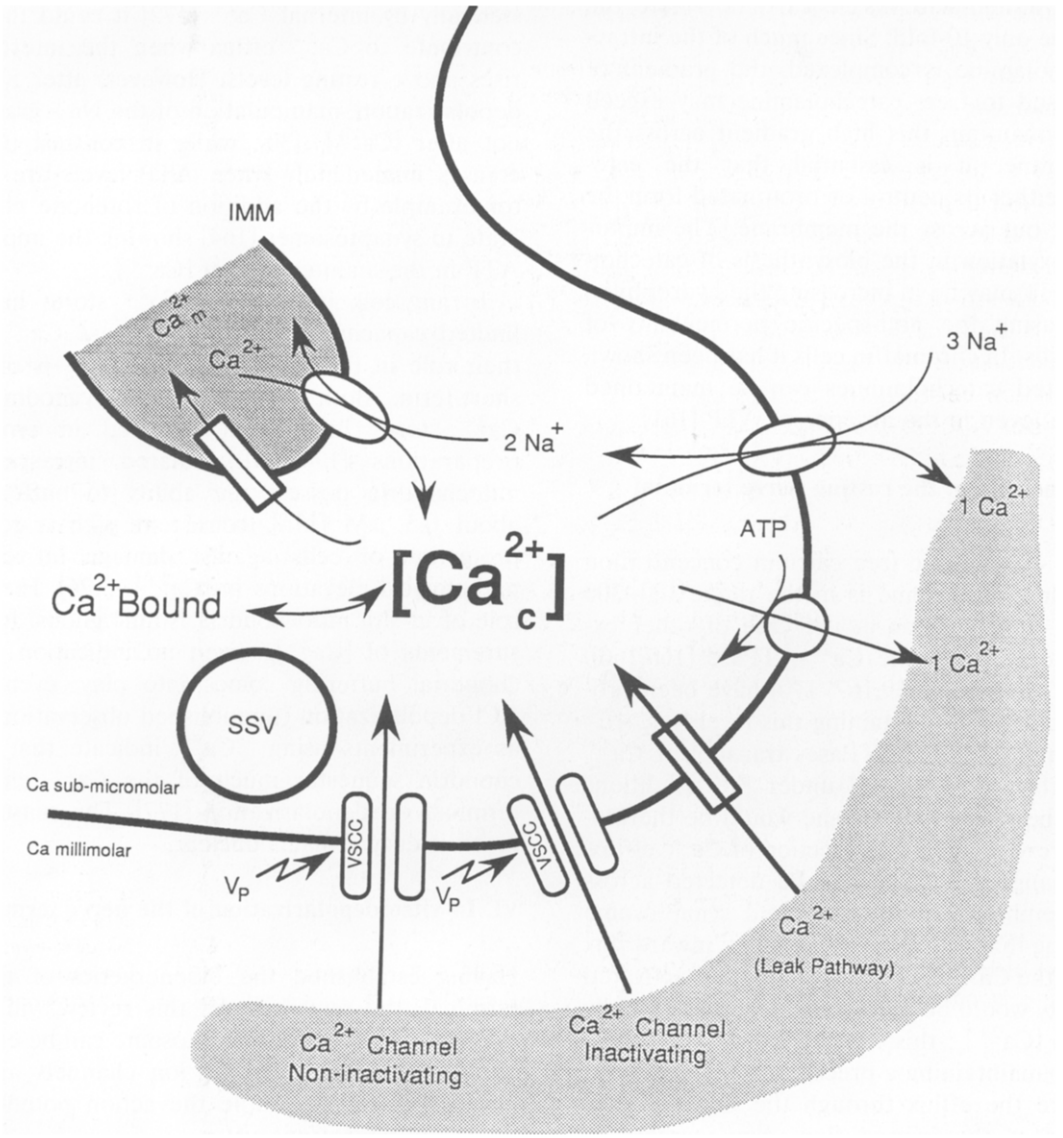

Fig. 4. Synaptosomal Ca^{2+} fluxes. The resting $[Ca^{2+}]_c$ is maintained largely by the kinetic balance between the leak pathway and the Ca^{2+}-ATPase. On depolarization, Ca^{2+} enters terminals through both inactivating and non-inactivating voltage-sensitive Ca^{2+}-channels. The Na^+–Ca^{2+} exchanger may contribute to the extrusion of elevated Ca^{2+}. Mitochondria play only a limited role in physiological $[Ca^{2+}]_c$ regulation, but can sequester massive amounts of Ca^{2+} when $[Ca^{2+}]_c$ is chronically elevated.

these data together, the probable mode of uptake is the inward transport of the cationic form of the catecholamine (the predominant form at neutral pH) in exchange for two protons, although it should be emphasized that it is not possible on thermodynamic grounds to distinguish this from exchange of neutral catecholamine for one proton.
The thermodynamics of this transport is given by:

$$\log_{10}\{[\mathrm{Tr}]_{in}/[\mathrm{Tr}]_{out}\} = \{\Delta\mathrm{pH}\}^2 + V_v/61.52 \quad (4)$$

Given a pH gradient of 1.5 units (inside acid) and a membrane potential of 50 mV (inside positive), this can maintain an equilibrium concentration gradient of free catecholamine ≥ 1000, i.e., far higher than that observed for the amino-acid vesicle carriers, where the gradient may be only 10-fold. Since much of the intravesicular catecholamine is complexed, the gradient of total, as opposed to free, catecholamine may exceed this value. To maintain this high gradient across the vesicle membrane, it is essential that the catecholamine, in either its neutral or protonated form, be unable to leak out across the membrane. The importance of hydroxylation in the biosynthesis of catecholamines and 5-HT may lie in increasing the hydrophilicity and decreasing the non-specific permeability of these compounds. In chromaffin cells it has been shown that accumulated catecholamines can be maintained for at least 1 h, even in the absence of ATP [161].

V. Ca^{2+} homeostasis in the resting nerve terminal

The resting cytoplasmic free calcium concentration ($[Ca^{2+}]_c$) in the synaptosome is in the range 100–300 nM [34,39,162–164] and, as in the case of other excitable membranes, both the Ca^{2+}-ATPase [165,166]) and Na^+–Ca^{2+} exchange [39,167–170] have been proposed to participate in maintaining this level (Fig. 4).

Plasma membrane Ca^{2+}-ATPases transport 1 Ca^{2+} per ATP hydrolyzed [171] and under the conditions pertaining at the plasma membrane would be thermodynamically irreversible in the direction of Ca^{2+} efflux. A steady exchange of $^{45}Ca^{2+}$ can be detected across the plasma membrane of the polarized synaptosome [166], suggesting that there is a constitutive inward flux of Ca^{2+}. Since the Ca^{2+}-ATPase is calmodulin-activated [171], its activity would be predicted to increase rapidly in response to $[Ca^{2+}]_c$; this would provide a sensitive mechanism for maintaining a precise resting $[Ca^{2+}]_c$ at the level where the efflux through the Ca^{2+}-ATPase precisely balances the inward flux. Any increase in $[Ca^{2+}]_c$ would be countered therefore by a net efflux from the terminal [166].

The 3 Na^+–Ca^{2+} exchanger may also contribute to $[Ca^{2+}]_c$ regulation in the terminal [39,167–170,172,173]. The maximum gradient of Ca^{2+} which can be maintained by a 3 Na^+–Ca^{2+} exchanger is given by:

$$\log_{10}\{[Ca^{2+}]_{out}/[Ca^{2+}]_{in}\} = \{3V_{Na} - V_p\}/61.52 \quad (5)$$

where V_{Na} and V_p are the equilibrium potential for Na^+ and the plasma membrane potential, respectively. For the polarized synaptosome, where these potentials are approx. +47 mV and −70 mV, respectively, the equilibrium Ca^{2+} gradient is 2700, which with an external Ca^{2+} of 1.3 mM corresponds to a $[Ca^{2+}]_c$ of 480 nM, i.e., very much at the upper limit of the observed $[Ca^{2+}]_c$. in any case, since the carrier is close to thermodynamic equilibrium it would be very inefficient as a means of regulating the resting $[Ca^{2+}]_c$.

Since the Na^+–Ca^{2+} exchanger is activated allosterically by internal Ca^{2+} [172] it could theoretically contribute to Ca^{2+} efflux when the internal $[Ca^{2+}]$ rises above resting levels. However, after KCl-evoked depolarization, manipulation of the Na^+ gradient does not alter $[Ca^{2+}]_c$ [30], while in contrast $[Ca^{2+}]_c$ increases immediately when ATP levels are collapsed, for example by the addition of rotenone plus iodoacetate to synaptosomes [164] showing the importance of ATP in the maintenance of $[Ca^{2+}]_c$.

In any cell, intracellular Ca^{2+} stores have only a limited capacity to store and release Ca^{2+} and thus their role in the regulation of $[Ca^{2+}]_c$ is of necessity short-term. Both (1,4,5)IP_3- and ryanodine-sensitive Ca^{2+} stores have been detected in synaptosomal preparations [174,175]. Isolated intrasynaptosomal mitochondria possess the ability to buffer Ca^{2+} at about 0.5 μM [176], consistent with a role in the protection of cells against damage in response to pathological elevations in $[Ca^{2+}]_c$ [176]. However, the role of in situ mitochondria is ambiguous: fura-2 measurements of $[Ca^{2+}]_c$ given no indication that mitochondrial buffering comes into play, even following KCl depolarization (unpublished observations), whereas experiments using $^{45}Ca^{2+}$ indicate that the mitochondria sequester much of the Ca^{2+} entering the terminal on depolarization [177]. The reason for this inconsistency remains unclear.

VI. In vitro depolarization of the nerve terminal

Having established the bioenergetics of the resting terminal, the remainder of this review will deal with the ways in which the synaptosome can be exploited to obtain information on the ion channels and release mechanism which couple the action potential to the release of neurotransmitter.

Physiological depolarization of an intact nerve terminal is induced by an action potential which is propagated down the axon opening Na^+-channels and allowing the influx of Na^+. The depolarization opens presy-

naptic Ca^{2+}-channels which trigger the release of transmitter (Fig. 5). The Na^+-channels inactivate and the membrane potential is restored with the involvement of Ca^{2+}-activated K^+-channels and delayed rectifying K^+-channels.

Since the synaptosome has lost its axon an alternative to the action potential is required to trigger release. Possible stimuli include depolarization induced by an increase in Na^+ conductance, an increase in K^+ concentration or a decrease in K^+ conductance or alternatively a direct elevation of $[Ca^{2+}]_c$ by addition of a Ca^{2+} ionophore. It is fair to say that difficulties in reproducing the physiological pattern of nerve terminal depolarization during the action potential have constituted the greatest single limitation of the synaptosomal preparation; however, as we shall discuss, much of this problem can now be overcome.

VI-A. Depolarization by preventing Na^+-channel inactivation

Even in populations of polarized synaptosomes voltage-activated Na^+ channels occasionally fire; this is seen from the action of the Na^+ channel inhibitor tetrodotoxin, which significantly lowers the basal $[Ca^{2+}]_c$ [30] and inhibits the dissipative Na^+ cycling between the Na^+ channel and the (Na^+/K^+)-ATPase, decreasing the basal respiration [178]. Normally an individual channel will remain open for only 1–2 ms; however, inactivation can be prevented by the alkaloid veratridine, with the result that in a few seconds all the channels are trapped in the open state, giving a large increase in Na^+ conductance and hence a permanent depolarization [28,179].

As a means of mimicking physiological depolariza-

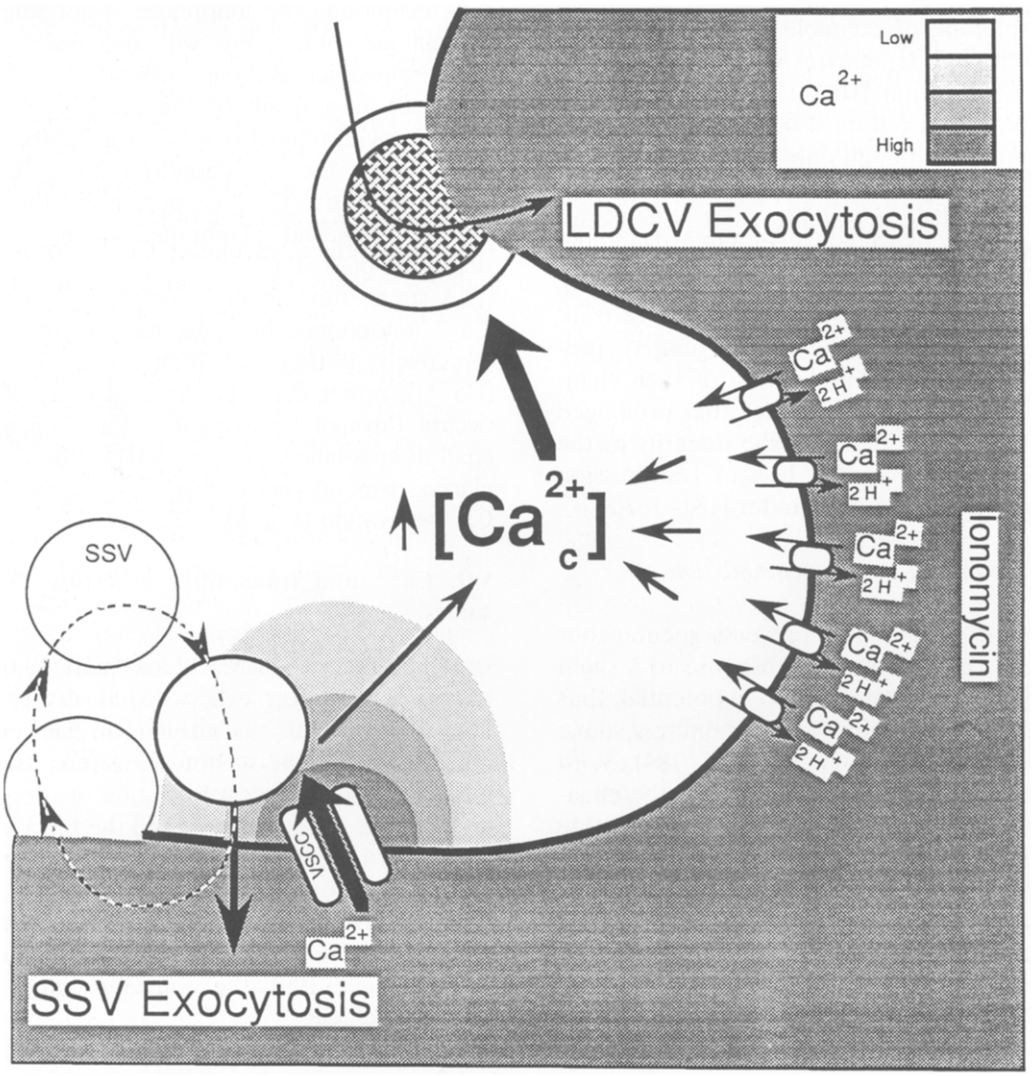

Fig. 5. Distinctive Ca^{2+}-secretion coupling mechanisms for small and large dense-core synaptic vesicles. Amino acids are released by Ca^{2+} entering through non-inactivating voltage-sensitive Ca^{2+} channels which appear to be directly coupled to the exocytotic apparatus for SSVs. Ca^{2+} subsequently diffuses into the bulk cytoplasm where it elevates $[Ca^{2+}]_c$. Bulk cytoplasmic $[Ca^{2+}]_c$ directly releases LDCVs. The $Ca^{2+}/2H+$ ionophore ionomycin which elevates $[Ca^{2+}]_c$ uniformly, is therefore effective in releasing LDCVs, but can release SSVs only when added in such a concentration that the entire terminal is flooded with a very high $[Ca^{2+}]_c$.

tion, this technique has three limitations: firstly it produces a 'clamped' depolarization, unlike the transient physiological action potential. Secondly, the continuous Na^+ entry causes a large increase in the activity of the (Na^+/K^+)-ATPase, causing a major energy drain and decreasing ATP levels unless the (Na^+/K^+)-ATPase is inhibited in parallel by ouabain [179]. A third effect of the collapse of the Na^+-electrochemical potential across the plasma membrane is a dramatic reversal of Na^+-coupled transport processes (see subsection III-B), and an extensive Ca^{2+}-independent efflux of metabolic pools of amino-acids, such as glutamate, GABA and aspartate from the cytoplasm [68,180], which greatly complicates the study of authentic exocytotic release.

VI-B. Depolarization by elevated KCl

Because of the dominance of the K^+ conductance, the plasma membrane of excitable cells approximates to a K^+-electrode and depolarizes when external $[K^+]$ is elevated (see subsection III-A). This large K^+ conductance of a polarized cell must be due to a noninactivating K^+ channel (although only transient K^+ channels have been detected electrophysiologically). Although elevated KCl is a standard and widely employed technique for depolarizing, it does have some severe limitations. Since KCl causes a single, clamped, depolarization and does not involve the firing of transient Na^+ or K^+ channels, one cannot observe processes which rely upon the modulation of such channels. Secondly, there is some evidence that prolonged exposure to elevated KCl damages the integrity of the terminal, leading to irreversible loss of transmission and non-synaptic release of transmitter [181–183].

VI-C. Depolarization by K^+-channel inhibition

The K_A-channel (see above) prevents spontaneous firing of terminals by activating in response to a slight depolarization and reversing the drop in potential, thus regulating neuronal excitability by maintaining a stable membrane potential (for review see Ref. 184). With synaptosomes and brain slices, inhibition of the channels by either nanomolar dendrotoxin (a polypeptide neurotoxin from the green mamba [185,186]) or millimolar 4-aminopyridine (4AP) appears to cause the terminals to undergo spontaneous action potentials, involving the tetrodotoxin-sensitive repetitive firing of Na^+-channels, as though the terminals were still receiving action potentials down its severed axon [30,62–64,187–192]. Thus 4AP and dendrotoxin more closely mimic the physiological mechanism of terminal depolarization than does elevated KCl or veratridine.

A variation on this depolarization technique is the use of Ba^{2+}, which also blocks K^+ channels and causes a tetrodotoxin-sensitive release of transmitter (unpublished data). In this case, there is no need for external Ca^{2+}, since Ba^{2+} entering through Ca^{2+}-channels is competent to trigger release [193–195].

It is notable that a number of presynaptic regulatory events which rely on modulation of transient ion channels are apparent only when synaptosomes are excited by K_A^+ channel blockade rather than with KCl or veratridine. For example, an enhancement of glutamate exocytosis with phorbol ester pretreatment is observed only with release evoked by 4AP and not with KCl-depolarization [196].

VI-D. Ca^{2+} ionophores

$Ca^{2+}/2H^+$ antiport ionophores such as A23187 and ionomycin are extensively employed to induce exocytosis from synaptosomes and other neuronal preparations. There are, however, a number of pitfalls with this technique. The ionophore is not selective for the plasma membrane, but will also insert into internal membranes such as the mitochondrion [197]. As $[Ca^{2+}]_c$ increases as a result of the ionophore action at the plasma membrane this sets up a dissipative Ca^{2+} cycling across the mitochondrial inner membrane between the native Ca^{2+} uniporter and the ionophore in the mitochondrial membrane, leading to an extensive depletion of ATP [197].

A more fundamental complexity with the use of Ca^{2+} ionophores, but one that can be exploited constructively, is that they induce a uniform increase in $[Ca^{2+}]_c$, rather than the directed entry of Ca^{2+} which occurs through voltage-gated Ca^{2+} channels. As we shall discuss below (Section VIII), different transmitter classes respond very differently to these two modes of Ca^{2+} elevation (Fig. 5).

VII. Ca^{2+} and transmitter exocytosis from synaptosomes

It has been recognized for many years that Ca^{2+} triggers transmitter exocytosis; however, a molecular understanding this the mechanism has remained stubbornly elusive. The synaptosome possesses all the apparatus to link the depolarization of the plasma membrane to the influx of Ca^{2+} and the release of transmitter and so in principle contains the elements which are required to address this fundamental problem. Apart from the protein chemistry of release, which will not be dealt with here since it is the subject of several recent reviews [5,198,199], the questions which can be addressed include:

(a) What is the nature of the presynaptic Ca^{2+} channels?

(b) How are they linked to transmitter exocytosis?

(c) Are different transmitters released by different mechanisms?

(d) What are the energetic requirements of exocytosis?

VII-A. Presynaptic Ca^{2+}-channels

Ca^{2+} channel terminology has largely followed the classic study of Nowycky et al. [200] in chick dorsal root ganglion cell bodies, which distinguished three subtypes of channel, termed T, L and N on electrophysiological grounds (for review see Ref. 201).

VII-A.1. Synaptosomal Ca^{2+} fluxes in response to depolarization

Due to the small size of the nerve terminal, electrophysiological studies have been impracticable, except in special cases (see below), and Ca^{2+} channels have therefore been investigated by monitoring $^{45}Ca^{2+}$ fluxes or fura-2 signals within the synaptosomes in response to depolarization, together with their pharmacological modulation and their coupling to transmitter release. $^{45}Ca^{2+}$ entry in response to prolonged KCl depolarization is biphasic, with an initial rapid entry of $^{45}Ca^{2+}$ followed by a slow uptake which shows no inactivation for the duration of the depolarization [167,202–210]. This is reflected in the fura-2 response, where an initial spike is followed by a sustained plateau [30,37,38,40, 170,180].

The transient entry can be largely inactivated by pre-depolarization of synaptosomes in the absence of external Ca^{2+}, such that when Ca^{2+} is readded, entry only occurs through the non-inactivating pathway [40,204,206,211–213]. It has been suggested that the non-inactivating phase represents the reversal of the Na^+/Ca^{2+} exchanger following depolarization [167, 168,214]. However, the fura-2 plateau persists in low Na^+ media [30] and so cannot be due to Na^+–Ca^{2+} exchange but to non-inactivating Ca^{2+}-channels.

The L-, T- and N-classification of Ca^{2+} channels is derived from studies on neuronal cell bodies and it does not necessarily follow that the channels at the presynaptic active zone which are responsible for triggering exocytosis conform to this classification. The evidence for and against the presence of 'classical' cell-body Ca^{2+} channels on synaptosomes will now be reviewed.

VII-A.2. Presynaptic L-type Ca^{2+}-channels

The neuronal L-channel is fundamentally similar to the thoroughly investigated skeletal muscle channel (for review see [215]). The neuronal L-channels are localized at the cell body [216] and are clustered at the base of major dendrites [217]. Although presynaptic terminals possess high-affinity binding sites for dihydropyridine antagonists of L-type Ca^{2+}-channels [208,210,218–220], most workers have found that Ca^{2+} fluxes and transmitter release are insensitive to the inhibitors [173,202,210,213,219–221], although a partial dihydropyridine sensitivity has also been reported for the initial rapid phase of ^{45}Ca uptake [208,222]. High concentrations of verapamil inhibit synaptosomal Ca^{2+} channels, although the effect is non-specific and non-stereoselective [213] and includes a blockade of Na^+-channels [202]. The dominant view is therefore that L-channels play little role in the presynaptic release mechanism, at least for Type 1 and Type 2 transmitters.

VII-A.3. Presynaptic T-type Ca^{2+}-channels

T-channels are the least well characterized of the cell body Ca^{2+} channels, in part because they lack specific inhibitors. The channels have a small conductance, are activated by a slight depolarization and inactivate rapidly. We are aware of no evidence for their presence on the synaptosomal membrane, although indirect electrophysiological evidence has suggested the presence of presynaptic low threshold Ca^{2+} channels [223].

VII-A.4. Presynaptic N-type Ca^{2+}-channels

N-type channels were so named because of their restriction to neuronal preparations [200]. They can be identified electrophysiologically as high-threshold (i.e., requiring an extensive depolarization), partially inactivating and dihydropyridine-insensitive. It is apparent that this classification has some limitations, and certainly encompasses a heterogeneous set of channels. Although mammalian synaptosomes have high affinity binding sites for Ω-conotoxin (GVIA) [224–227] which have been partially characterized [228], only a partial blockade of KCl-evoked $^{45}Ca^{2+}$ entry has been reported, even at concentrations much higher than those which inhibit the cell-body N-channels [173,213,229]. In contrast, the initial phase of KCl-evoked ^{45}Ca entry into synaptosomes from chick or frog brain is much more sensitive to conotoxin, total inhibition being observed at 0.3 μM [230].

VII-B. Voltage-activated Ca^{2+}-channels linked to transmitter release

General Ca^{2+}-channel inhibitors such as Ruthenium red and La^{3+} can inhibit all transmitter release from terminals, probably by non-selective charge interactions [231]. Ruthenium red is a hexavalent cation which inhibits the Ca^{2+}-uniporter in mitochondrial membranes [25,232]. It does not cross the plasma membrane of intact synaptosomes [233] but inhibits plasma membrane Ca^{2+}-channels [231] and the release of GABA [234] and ACh [235,236]. La^{3+} inhibits depolarization induced $^{45}Ca^{2+}$ uptake [39,209] and Ca^{2+}-dependent release of glutamate [237,238], dopamine [239] and GABA [240].

It is essential to be aware of the possibility that different classes of neurotransmitters may be released by different mechanisms and that transmitter-specific subclasses of release-coupled Ca^{2+} channels might ex-

ist, or even that the channel type for a given transmitter might differ in different brain areas and different species. For this reason we shall consider the evidence for each transmitter class separately, and confine our discussion to mammalian terminals.

VII-B.1. Amino-acid release

While no specific inhibitors are known which can distinguish the two phases of voltage-dependent Ca^{2+} entry into KCl-depolarized synaptosomes (subsection VII–B), there is a rapid voltage-dependent inactivation of the initial phase of $^{45}Ca^{2+}$ entry following KCl-depolarization [204,206,211–213]. Thus, predepolarization of synaptosomes in the absence of external Ca^{2+} allows this component to be selectively inhibited without affecting the subsequent non-inactivating 'plateau' phase when Ca^{2+} is re-added. The release of transmitter GABA [241] and glutamate [40] from synaptosomes is unaffected by this inactivation, indicating that their release is coupled to the non-inactivating component.

A non-inactivating Ca^{2+}-channel is consistent with presynaptic patch-clamp analysis of the large hippocampal mossy fibre terminals from the cerebellum, which can be purified on the basis of their large size [17,242]. These terminals release glutamate and prodynorphin-derived peptides [17] and are just large enough for patch-clamp analysis of currents. Taken together, this evidence indicates that the mammalian presynaptic Ca^{2+} channels associated with amino-acid exocytosis may be predominantly non-inactivating and resistant to dihydropyridines and conotoxin, although a combination of L- and N-channel antagonists was found to block GABA release from cultured striatal neurones [243].

VII-B.2. Type 2 transmitter release

In contrast to the amino-acids, catecholamine exocytosis from synaptosomes has been reported to correlate with the initial rapid phase of Ca^{2+} entry [204,244,245]. Similarly, the more rapid phase of acetylcholine release from KCl-depolarized synaptosomes correlates with the initial phase of Ca^{2+} entry [163,246]. The possibility must therefore be addressed that amino-acids and biogenic amines are released by distinctive Ca^{2+} channels. Consistent with this, Ω-conotoxin (GVIA) causes a potent inhibition of release of acetylcholine [247], noradrenaline [213,247–252], dopamine [229] and 5-HT [213], suggesting their linkage to presynaptic N-channels. This is not inconsistent with the failure to observe a convincing inhibition of Ca^{2+} entry into mammalian synaptosomes with conotoxin, since catecholaminergic synaptosomes represent only a minor fraction of terminal preparations from most brain areas.

VII-B.3. Peptide release

As will be discussed below (subsection VII-D), the mechanism of neuropeptide release from terminals is distinctive, relying on a bulk increase in $[Ca^{2+}]_c$ rather than a localized coupling between Ca^{2+} channels and exocytosis. The presynaptic Ca^{2+}-channels on the unusually large synaptosomes from rat pituitary have been examined electrophysiologically and have properties characteristic of N-channels [253]. Another large terminal preparation is obtained from the neurohypophysis [116,254]. These terminals are 1–10 μm in diameter and secrete peptide neurohormones (oxytocin and vasopressin – contained in LDCVs) into the blood. The terminals also contain SSVs with a protein composition similar to the SSVs of other terminals, but their function is unknown [116]. Patch-clamp analysis has revealed two types of Ca^{2+}-channel, an L-type and a high-threshold N-like current [255]. It has been been proposed that the N-channels are coupled to peptide secretion [256].

VIII. The coupling of Ca^{2+} to exocytosis in synaptosomes

It is evident that there is no universal mechanism for exocytosis which encompasses the sub-millisecond release of amino acids in the brain, the release of biogenic amines (delay of tens of milliseconds), neuropeptide release (longer delay) and the slow secretion of hormones in non-neuronal systems. As the previous section indicates, even within the brain the release of different classes of neurotransmitters appear to be coupled to distinctive Ca^{2+} channels. It is therefore essential to consider each exocytotic process separately.

The sub-millisecond delay between depolarization and fast-acting neurotransmitter release puts severe constraints on the properties that this hypothetical entity must possess.

(1) There is no time for Ca^{2+} to diffuse and equilibrate with the cytoplasm, thus the theoretical calculation of Simon and Llinas [257] places an upper limit on the distance between the Ca^{2+}-channel and the Ca^{2+}-sensor of 100 nm, or 2 times the diameter of a synaptic vesicle.

(2) The frequency with which repetitive nerve stimulation can occur (at least 10 Hz) requires that Ca^{2+} must rapidly dissociate from the trigger. This requires both that the binding affinity of the trigger for Ca^{2+} must be low (the definition of a fast off-rate) and that some means must exist for lowering $[Ca^{2+}]$ in the region of the trigger with extreme rapidity.

(3) There is no time for vesicles to diffuse to the membrane, thus it must be assumed that some vesicles are already docked at the release site.

(4) There is no time for covalent modification such as phosphorylation to occur [258].

Since the temporal and spatial resolution of biochemical experiments with synaptosomes is limited, it

might be thought that this preparation could not contribute to our understanding of the release mechanism. However, it is possible to show that there is a close coupling between Ca^{2+} entry through the voltage-activated Ca^{2+} channels and glutamate release (see Fig. 5). When the release of glutamate evoked by either KCl or 4AP is plotted as a function of the bulk cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_c$, measured by fura-2 a very steep relationship is obtained, with zero exocytosis at resting $[Ca^{2+}]_c$ (about 200 nM) and maximal release at a plateau $[Ca^{2+}]_c$ of 400 nM [40,41]. This means either that release is triggered by a small increase in bulk $[Ca^{2+}]_c$, or that fura-2 is not accurately measuring $[Ca^{2+}]$ at the trigger site where a large signal from Ca^{2+} entry in this area would be masked by a lower signal from the bulk cytoplasm.

These alternatives can be distinguished by inducing a uniform increase in $[Ca^{2+}]_c$ with the $Ca^{2+}/2H^+$ ionophore ionomycin (see Fig. 5). For the same average $[Ca^{2+}]$ increase measured by fura-2, Ca^{2+} entry through voltage-activated Ca^{2+}-channels was 8-times more effective than the ionophore at releasing glutamate [40,41]. Since plasma membrane potential has been proposed to play an integral role in the release process [259,260] the experiment was repeated after depolarization in the presence of high $[Mg^{2+}]$ to inhibit the voltage-activated Ca^{2+} channels: again ionomycin was almost ineffective in releasing the amino acid, indicating an intimate relationship between the directed Ca^{2+} entry through the channels and the release of amino acid [41].

When the release of a representative neuropeptide, cholecystokinin-8, was determined from the same preparation, ionomycin was highly effective and no advantage was gained from allowing Ca^{2+} entry to occur through voltage-gated Ca^{2+} channels [41]. Neuropeptides are released from large dense-core vesicles (LDCVs), and the electron microscope reveals that these vesicles are released at apparently random sites rather than at the highly organized 'active zone' directly apposed to the post-synaptic receptors [22,23]. Furthermore, neuropeptides are released only by high-frequency stimulation of terminals, rather than by single action potentials [120]. These observations both suggest that neuropeptides, in contrast to amino acids, are released by bulk cytoplasmic $[Ca^{2+}]_c$ (see Fig. 5).

A close proximity between the presynaptic Ca^{2+}-channels and the trigger has kinetic advantages both for the initiation and the termination of exocytosis. Onset of release can be very rapid, because the Ca^{2+} has only a very short distance to travel after emerging from the mouth of the channel, and because it is not necessary to raise $[Ca^{2+}]_c$ in the entire terminal. Secondly, since $[Ca^{2+}]_c$ in the vicinity of the channel mouth can be very high, say 10–100 μM, the affinity of the trigger for Ca^{2+} can be relatively low, which has the kinetic advantage that the release will be terminated quickly when the Ca^{2+}-channel closes, since a low affinity means a rapid rate constant for dissociation. Furthermore, when the Ca^{2+}-channel closes, Ca^{2+} will diffuse away from the mouth of the channel, and the local concentration will fall within milliseconds to a fraction of the peak value [261]. Thus, the termination of release does not rely on the need to pump bulk Ca^{2+} out of the cytoplasm, either across the plasma membrane or into internal organelles.

IX. Ca^{2+}-independent release of neurotransmitters

In vitro depolarization of synaptosomes, brain slices or cultured neurones can result in a Ca^{2+}-independent release of transmitter which can be observed in isolation by incubation in a Ca^{2+}-free medium and which is superimposed on the Ca^{2+}-dependent release in a Ca^{2+}-containing medium. Some Ca^{2+}-independent release can be observed with most transmitters, but the effect is most prominent in the case of the amino acids and correlates with the duration and extent of the collapse of the Na^+ electrochemical potential across the plasma membrane [68]. Thus, little Ca^{2+}-independent release is associated with the action potentials induced by 4AP in synaptosomal preparations [30]; release becomes significant with prolonged KCl-evoked depolarization (where V_p is collapsed but the Na^+ concentration gradient is retained) and is most extensive following veratridine (when both V_p and the Na^+ concentration gradient are collapsed). This Ca^{2+}-independent release is due simply to the reversal of the electrogenic Na^+-coupled uptake carriers for the transmitters as the Na^+-electrochemical potential gradient for the ion decreases.

The cytoplasmic origin for the released transmitter under these circumstances is supported by the co-release of endogenous or radio-labelled aspartate [180], which can be regarded as a marker of cytoplasmic glutamate, since it cannot be accumulated significantly into synaptic vesicles (see Section IV). In contrast to Ca^{2+}-dependent exocytotic release, which is inhibited by a drop in ATP (see below), Ca^{2+}-independent release does not require ATP, and is actually increased under conditions of energy deprivation, due to a decline in the Na^+ gradient [164].

This efflux must always be taken into consideration in vitro, although in vivo negligible GABA or glutamate would be lost from the cytoplasm in the millisecond time-scale of physiological depolarization. In vitro the relative contribution of Ca^{2+}-independent release can be minimized by measuring efflux only during the first 1–3 min of depolarization, or by substituting choline for Na^+ prior to KCl depolarization [262].

Although it has been suggested that this mode of efflux could be significant during the normal duration

of a physiological action potential (see, for example, Ref. 67), it is much more likely that the Ca^{2+}-independent release which is seen in vitro is due to the unphysiologically prolonged depolarization, and that the only in vivo conditions where large amounts of transmitter are released from the cytoplasm by reversal of the Na^+-coupled uptake pathway are those where the Na^+-electrochemical potential is chronically depressed, as in ischaemia, which will now be discussed.

IX-A. Glutamate release in brain ischaemia

Hypoxia, hypoglycaemia and ischaemia are the clinical terms applied to conditions where either the supplies of oxygen, glucose or both oxygen and glucose are respectively terminated. The brain is particularly sensitive to these conditions and an understanding of the bioenergetics of transmitter accumulation and release has now begun to unravel the sequence of events that occur. Reduced oxygen supply to the brain while maintaining bloodflow (hypoxia) occurs in respiratory arrest, carbon monoxide poisoning, or near-drowning. The brain is highly dependent on glucose, and hypoglycaemia, as in insulin overdose, can lead to coma. In insulin-induced hypoglycaemia, ketone bodies do not provide an energy source for the brain, as they do in starvation (see Ref. 263). Complete disruption of the blood supply to the brain (ischaemia) occurs following cardiac arrest (global ischaemia), while in stroke the disruption can be localized to particular areas (focal ischaemia). Common to hypoxia, hypoglycaemia and ischaemia is a fall in energy levels. However, much of the damage to the brain is due not to the energy deprivation per se, but to the neurotoxic effect of glutamate which is released in massive amounts under these conditions [50,52,263,264]. Glutamate neurotoxicity is largely due to an excessive stimulation of the *N*-methyl-D-aspartate (NMDA) selective subclass of glutamate receptors and may be mediated by a massive post-synaptic Ca^{2+} entry through this receptor [53, 265–268].

IX-B. In vitro models of hypoxia, hypoglycaemia and ischaemia

In vivo, an energy depletion will probably not effect all parts of the neurone equally. Because the presynaptic terminals of neurones have a very high metabolic rate they are likely to be more susceptible. The energetic effects of these conditions can be mimicked with isolated presynaptic terminals by using various combinations of inhibitors. Rotenone, inhibiting complex I of the mitochondrial respiratory chain, will have an energetic effect to hypoxia, while inhibition of glycolysis by iodoacetate or by glucose deprivation will mimic hypoglycaemia. A combination of the two inhibitions will mimic the more extreme condition of ischaemia.

As discussed above (Section III) the addition of rotenone or a protonophore to synaptosomes increases glucose utilization 10-fold [26,57]. However, this does not totally compensate for the loss of oxidative phosphorylation, since overall ATP/ADP ratios are somewhat lowered [26,57]. Even if glucose supply is retained, e.g., in anoxia, it is likely that terminal glycolysis will become inhibited since, for reasons which are not clearly understood, synaptosomal glycolysis fails after a few minutes of experimental anoxia induced by N_2 or CN^-, conditions under which the respiratory chain becomes very reduced, but is maintained indefinitely after rotenone, when the respiratory chain components remain oxidized [57]. In synaptosomal incubations, the reduced ATP level resulting from respiratory or glycolytic inhibitors leads to a reduced Na^+/K^+-ATPase activity and a progressive depolarization and lowering of the Na^+ electrochemical gradient across the plasma membrane [26,164]. Accompanying this is a release of glutamate [269,270], which however has a cytoplasmic origin since it is accompanied by a parallel release of aspartate and is Ca^{2+}-independent [164,180]. At the same time, Ca^{2+}-dependent release of glutamate is inhibited due to the fall in ATP levels. The release of transmitter during these conditions has been attributed to a Ca^{2+}-dependent exocytosis of synaptic vesicles [271] consequent upon an increase in $[Ca^{2+}]_c$ due to reduced activity of the plasma membrane Ca^{2+}-ATPase. However, it is possible to resolve the time-course of the different effects in synaptosomes: the inhibition of Ca^{2+}-dependent glutamate release occurs immediately the ATP level drops, and well before $[Ca^{2+}]_c$ has increased to levels which could conceivably evoke glutamate exocytosis [164], even discounting the relative ineffectiveness of a uniform increase in Ca^{2+} rather than one directed through voltage-activated Ca^{2+} channels.

X. Conclusions

We have attempted in this review to demonstrate that the synaptosomal preapration can yield a wealth of information on the mechanism and regulation of transmitter uptake, storage and release. Future advances will require a close integration between functional studies, such as are reported here, and information on the role of terminal-specific proteins, in order to build up a complete picture of the exocytotic/endocytic cycle in the nerve terminal.

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