Storage and release of acetylcholine by a clonal cell line
(dense-core vesicle/sympathetic neuron)

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ABSTRACT. The nerve cell line PC12, in its morphologically undifferentiated state, synthesizes, stores, and secretes catecholamines and acetylcholine. At least 60% of the basal level of neurotransmitter release is due to a calcium-dependent mechanism, and the rate of secretion is enhanced by increasing external potassium. A minimum of 90% of the intracellular acetylcholine and catecholamines are stored in particulate structures. The storage site for acetylcholine is dense core vesicles that can be distinguished from those containing catecholamines on the basis of vesicle density on sucrose gradients, vesicle size, and reseptor sensitivity. These results are discussed in relation to what are thought to be the early stages of synapse formation in cell culture.

A clonal cell line was recently isolated which shares many properties with primary cultures of sympathetic ganglion neurons. This clone, designated PC12, responds to exogenous nerve growth factor (NGF) by the extension of neurites (1), and synthesizes catecholamines (1) and acetylcholine (ACh) (2). In addition, the cells form cholinergic synapses with a clonal cell line of skeletal muscle (2), and miniature endplate potentials (mepps) have been detected within 1 hr after mixing PC12 and skeletal muscle cells in the absence of exogenous NGF (Y. Kidokoro, personal communication). Due to the rapidity of synapse formation and the large amounts of both ACh and catecholamines synthesized by the PC12 clone, these cells are ideally suited for study of the storage and release of the neurotransmitters and the relationship of these processes to the early stages of synapse formation. The following experiments show that neurotransmitters are continually released by morphologically undifferentiated PC12 cells and that ACh is stored in a dense core vesicle distinct from the 30- to 50-nm clear vesicles normally associated with cholinergic synapses.

MATERIALS AND METHODS

The clonal rat cell line PC12 was obtained from Lloyd Greene (1). The cells were grown on plastic tissue culture dishes in 10% fetal calf and 5% horse serum as described (2). They were dissociated by vigorous pipetting for passaging and for all of the following experiments. To assay ACh and catecholamine synthesis, storage, and release, cells in the late exponential growth phase were labeled for 20 hr (approximately one-half a generation time) with [3H]choline (5 μCi/ml; specific activity, 1 Ci/mmol), [14C]choline (2 μCi/ml; specific activity, 30 mCi/mmol), or [3H]tyrosine (5 μCi/ml; specific activity, 30 Ci/mmol) in Dulbecco’s modified Eagle’s medium containing 1% of the normal amount of tyrosine or choline, 10% fetal calf serum, and 5% horse serum. The cells were then washed in release buffer (0.01 M N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, pH 7.1/0.15 M NaCl/5 mM KC1/0.01 M glucose/2 mM CaCl2/1 mM MgCl2. 0.1 mM eserine) and treated as indicated. To assay neurotransmitter release, cells were incubated in release buffer with or without increased concentrations of potassium at 37° for the indicated times, the cells were pelleted by centrifugation, and aliquots of the supernatant were analyzed for ACh, norepinephrine (NE), choline, dopamine (DA), and tyrosine by high-voltage electrophoresis (3). For density gradient analysis, isotopically labeled washed cells were homogenized in 0.25 M sucrose/1 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, pH 7.0/0.1 mM eserine, and centrifuged on one of the following gradients in a Beckman SW 41 rotor: (i) 0.6–1.8 M sucrose linear gradient for 2 hr at 35,000 rpm (4); and (ii) a step gradient of 0.4, 0.8, 1.2, and 1.6 M sucrose at 25,000 rpm for 90 min (5). Isotopically labeled synaptic vesicles were prepared from rat cortex by the method of Richter and Marchbanks (6). All gradients contained 0.1 mM eserine.

To assay chromogranin secretion, cells were labeled for 20 hr with [3H]leucine (10 μCi/ml; specific activity, 10 Ci/mmol) or [14C]leucine (1 μCi/ml; specific activity, 50 Ci/mmol) in leucine-free modified Eagle’s medium containing 10% fetal calf serum and 5% horse serum. The cells were washed with release buffer and incubated at 37° in the presence of the desired reagents. After removal of cells by centrifugation, the supernatant was assayed for chromogranin A (CGA) and dopamine β-hydroxylase [DBH; 3,4-dihydroxyphenylalanine, ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1] by disc electrophoresis in sodium dodecyl sulfate. These two proteins were identified on the basis of their comigration on two dimensional acrylamide gels with DBH and CGA from adrenal medulla, comigration on density gradients with catecholamine-containing vesicles, and their potassium-induced release along with catecholamines. Electron microscopy was performed as described (7).

RESULTS

Neurotransmitter Release. Morphologically undifferentiated PC12 cells synthesize both catecholamines and ACh (2). If these cells possess a functional excitation-secretion coupling mechanism, then conditions that depolarize the cell should release the neurotransmitters. To assay for transmitter release, exponentially dividing cells were labeled with [3H]tyrosine or [3H]choline for 20 hr in growth medium containing decreased levels of these amino acids and 15% serum. After extensive washing, the secretion of ACh, choline, DA, and NE was followed as a function of time in the presence of 5 or 50 mM KCl. Fig. 1 shows that under normal conditions (5 mM KCl), the cells spontaneously released catecholamines and ACh. In the presence of elevated potassium levels, the rate of release increased and was dependent upon the concentration of external potas-
FIG. 1. Release of catecholamines and ACh from PC12 cells. Exponentially dividing cells were labeled for 20 hr with [3H]choline or [3H]tyrosine, washed, and resuspended in release buffer; the release of the neurotransmitters was followed as a function of time. DA and NE have indistinguishable secretion kinetics and their sum is presented in the data. NE constitutes a constant of 21% of the total secreted catecholamines (NE and DA). The data are presented as the percent of total intracellular neurotransmitter at time 0 that is released as a function of time. They are means of duplicate determinations, with a variation of less than 7%. The rate of release of tyrosine and choline was not appreciably increased over their spontaneous release rate by 50 mM KCl, X, Catecholamines, basal release; ○, ACh, basal release; ◦, catecholamines, 50 mM K+, ◦, ACh, 50 mM K+.

FIG. 2. The release of neurotransmitters and of DBH and CGA as a function of external potassium concentration. Cells were labeled for 20 hr with [3H]choline, [3H]tyrosine, [14C]leucine, or [3H]leucine, washed, and resuspended in release buffer containing the indicated concentrations of potassium; the release of neurotransmitters and of DBH and CGA was assayed after a 30-min incubation at 37°C. The data are plotted as the ratio of neurotransmitter released under the experimental condition to that of cells in normal release buffer. In the case of CGA and DBH, 14C-labeled secreted proteins from each potassium concentration were mixed with 3H-labeled protein from control cultures incubated under the same conditions in normal release buffer containing 5 mM potassium and lyophilized. After sodium dodecyl sulfate electrophoresis, the areas under the CGA and DBH protein radioactivity curves were determined and the data were plotted as the numerical ratio of the experimental to control values. The results were the same when the isotopic labels were reversed. (A) Neurotransmitter release. X, DA; ○, NE; △, ACh. (B) DBH and CGA release. ◦, CGA; ○, DBH.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Catecholamines, %</th>
<th>ACh, %</th>
<th>Choline, %</th>
<th>DBH, %</th>
<th>CGA, %</th>
<th>ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>6.1</td>
<td>8.0</td>
<td>65</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>K+, 50 mM</td>
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<td>24</td>
<td>75</td>
<td>2.5</td>
<td>2.7</td>
<td>2.7</td>
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<td>Ca2+</td>
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<td>6.4</td>
<td>60</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ca2+, K+ (50 mM)</td>
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<td>8.6</td>
<td>73</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>10 mM Mg2+</td>
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<td>3.0</td>
<td>57</td>
<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Ca2+, Mg2+ (10 mM)</td>
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<td>2.8</td>
<td>61</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>(50 mM)</td>
<td>3.1</td>
<td>3.5</td>
<td>70</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mg2+, 10 mM</td>
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<td>7.2</td>
<td>68</td>
<td>1</td>
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<td>1</td>
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<tr>
<td>Mg2+ (10 mM), K+</td>
<td>8.9</td>
<td>14</td>
<td>71</td>
<td>1.6</td>
<td>1.5</td>
<td>1.5</td>
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</tbody>
</table>

Cells were labeled for 20 hr with the appropriate precursors, washed, and incubated for 30 min at 37°C in release buffer; the secreted neurotransmitters and proteins were assayed. In the case of 50 mM K+, NE was replaced by K. The data are means of duplicate or triplicate determinations; the variation between replicates was less than 7%. The data for the catecholamines (DA plus NE), ACh, and choline are presented as the percent of total cellular transmitter present at time 0 that was released after 30-min incubation. DBH and CGA data are presented as the ratio of experimental to control values of released protein (see Fig. 2 for details). * Due to the high background of proteins on the acrylamide gels, ratios less than 1 cannot be reproducibly detected. + Normal release buffer.

is neurotransmitter release due to a physiological process. The first alternative would be ruled out if it were demonstrated that the basal level of release was decreased by reagents that are known to affect the calcium-dependent release mechanisms. The data in Table 1 show that either high magnesium or calcium deprivation inhibit the unstimulated release rate for neurotransmitters 10% and 80%, respectively. Cobalt, which competes with and blocks many calcium-mediated processes (8), decreases the basal level of release by >60%.

Intracellular Storage of ACh and Catecholamines. ACh and the catecholamines could be present either in vesicles or in the cytoplasm of the PC12 cells. To determine if these compounds are associated with a particulate subcellular fraction, cells were labeled for 18 hr with [3H]tyrosine and [14C]-choline. They were then washed twice with release medium, homogenized in 0.25 M sucrose containing 1 mM N-1-hydroxyethylpiperazine-N'-2-ethanesulfonic acid pH 6.9, and 0.1 mM eserine, and nuclei and unassembled cells were removed by centrifugation. As controls, one aliquot of the homogenate received Triton X-100 to a final concentration of 0.1%, [3H]ACh was added to another prior to homogenization. The samples were then centrifuged at 35,000 rpm in linear gradients from 0 to 1.8 M sucrose for 2 hr (4). Samples were collected and lyophilized, and the distribution of radioactivity in ACh, choline, tyrosine, NE, and DA was determined. More than 80% of the catecholamines and ACh banded with particles of similar but not identical densities in the gradient (Fig. 3A). The equilibrium density for catecholamines was approximately 1.2 and that of ACh, 1.0. Free tyrosine, choline, and the [3H]ACh added before homogenization as a control for trapping remained at the top of the gradient. The detergent Triton X-100 released the particulate neurotransmitters into the soluble fraction. Because the high density of ACh-containing particles was atypial (5), their density was compared with vesicles from brain cortex on a different gradient system. When a homogenate of [14C]choline-labeled PC12 cells and [3H]choline-labeled brain cholinergic vesicles was centrifuged on a step gradient
of 0.4, 0.6, 0.8, and 1.2 M sucrose, the ACh derived from PC12 was found in the bottom of the gradient and the rat brain cholinergic vesicles were at the initial 0.25–0.4 M interface (5).

Although their differences in buoyant density make it unlikely, it is possible that both ACh and catecholamines are stored in the same vesicle. For example, some vesicles could contain both ACh and catecholamines and others, only catecholamines. Alternatively, the two classes of transmitters could be stored in different vesicles. To distinguish between these possibilities, cells were labeled for 18 hr with [3H]choline and [3H]tyrosine in the presence or absence of 2 μM reserpine, a reagent known to deplete catecholamine storage vesicles (9). If the same vesicles contain both ACh and catecholamines, then reserpine should deplete both transmitters. Table 2 shows that reserpine blocked the accumulation of intracellular DA and NE by 97%, whereas ACh synthesis and storage was decreased less than 15%. In addition, the potassium-stimulated release of ACh was normal, and DBH and CGA were secreted in the presence of 50 mM K+ in the same absolute amount as in the potassium-stimulated control cultures. A similar result was obtained when the cells were labeled for 14 hr with radioactive transmitter precursors and treated with reserpine for 2 hr to deplete intracellular stores. Intracellular catecholamines were decreased by 90% and ACh by 11%; the remaining catecholamines and ACh were released by high potassium as were DBH and CGA. When the reserpine-treated cells are homogenized and run on a sucrose gradient, the catecholamine-containing particulate fraction was decreased by 97% and the ACh storage particles remained (Fig. 3B). After an 18-hr exposure to reserpine, the ACh-containing particulate fraction was slightly broadened and shifted one or two fractions to a lower density than in the control. These effects were not seen after a 2-hr exposure to reserpine, although the loss of ACh was similar (Table 2). The density alteration of the ACh-containing particles was probably due to a nonspecific effect of reserpine on vesicle structure. Finally, when the distribution of DBH and CGA in the gradient was assayed after extended reserpine treatment, the concentration of both proteins in the particulate neurotransmitter fractions was decreased to 18% of the control cultures.

If the above arguments are valid and there are two classes of vesicles within PC12 cells, then it should be possible to detect these differences morphologically. When PC12 cells grown in the absence of NGF were examined by electron microscopy, a large number of dense-core vesicles were observed. When vesicle diameter was plotted against frequency of appearance, there was a wide distribution of particle sizes, with a peak frequency at a diameter of 200 nm (Fig. 4). Because reserpine depletes the catecholamine content of PC12 cells, it follows that, if one size class of vesicles was eliminated by this compound, it represents the catecholamine-containing particles. Fig. 4 shows that the larger diameter vesicles were greatly decreased in frequency after reserpine treatment and that vesicles averaging 100 nm become the predominant class. The absolute number of dense-core vesicles per unit area of cytoplasm was decreased about 50% (Fig. 5). If the smaller vesicles contained ACh, then they should comigrate on a sucrose gradient with radioactive ACh after reserpine treatment. Reserpine-treated cells were homogenized and the vesicles were sedimented on a sucrose gradient as described in Fig. 3. Each gradient fraction was then fixed with gluteraldehyde and repelled. The fractions corresponding to the sedimentation position of ACh contained the 100-nm class of dense-core vesicles found in—

### Table 2. Effect of reserpine on the storage and release of neurotransmitter, CGA, and DBH

<table>
<thead>
<tr>
<th>Condition</th>
<th>Reserpine</th>
<th>NE</th>
<th>DA</th>
<th>ACh</th>
<th>CGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Reserpine</td>
<td>18 hr</td>
<td>0.03</td>
<td>0.02</td>
<td>0.87</td>
<td>—</td>
</tr>
<tr>
<td>Secreted:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Reserpine, K+</td>
<td>18 hr</td>
<td>ND*</td>
<td>ND*</td>
<td>0.9</td>
<td>1</td>
</tr>
<tr>
<td>Reserpine, K+</td>
<td>18 hr</td>
<td>ND*</td>
<td>ND*</td>
<td>3.0</td>
<td>3.1</td>
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<td>Intracellular:</td>
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<tr>
<td>Control</td>
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<td>0.09</td>
<td>0.11</td>
<td>0.89</td>
<td>—</td>
</tr>
<tr>
<td>Reserpine</td>
<td>2 hr</td>
<td>0.2</td>
<td>0.3</td>
<td>2.5</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Cells were labeled for 18 hr with [3H]tyrosine, [3H]choline, [3H]leucine, or [14C]leucine in the presence or absence of 2 μM reserpine. In some cases 2 μM reserpine was added 2 hr before the cells were assayed. The cells were then washed and resuspended in release buffer with or without 50 mM K+. After 30 min at 37°C, the secreted molecules were assayed. The data are presented as the ratio of experimental to control values. The absolute amount of DBH and CGA (fraction of total cellular isotope) secreted in the reserpine-treated cultures was the same as in the controls. Reserpine did not alter the incorporation of leucine into protein.

* Decreased basal secretion rate could not be detected in this assay.

† ND, amount too low to quantitate accurately.
serpine-treated cells, along with free ribosomes and very few other membranous structures (Fig. 6).

**DISCUSSION**

The following conclusions may be made from the above data. (i) Morphologically undifferentiated PC12 cells synthesize and secrete ACh, NE, DA, and two proteins characterized as DBH and CGA (Figs. 1 and 2; Table 1). (ii) Depolarizing the cells with potassium increases the rate of release of all of these molecules; the induced increases can be blocked by high magnesium, by removing external calcium, or by cobalt ions (Figs. 1 and 2; Table 1). (iii) The basal level of transmitter release is inhibited 60% by cobalt, showing that at least this fraction is due to a calcium-dependent physiological process (Table 1). (iv) Approximately 80% of ACh and the catecholamines is stored in particles with similar but not identical densities on sucrose gradients (Fig. 3). (v) ACh vesicles are also distinguishable from those containing DA and NE on the basis of their lack of sensitivity to reserpine (Table 2; Fig. 3B). (vi) Morphological evidence suggests that ACh is stored in 100-nm dense-core vesicles (Figs. 4, 5, and 6).

Although a spontaneous release of neurotransmitters has been reported in essentially all studies on evoked release, apparently little attention has been paid to this phenomenon. Perhaps the best studied example is the release of ACh by the phrenic nerve in the rat diaphragm (10). In this case the spontaneous release rate was 23% of the endogenous ACh store per 30 min, which is higher than the basal release level of 8% per 30 min observed with the PC12 clone (Fig. 1). The release of ACh and catecholamines from PC12, in the absence of experimental perturbation, could result from a random functioning of the normal release mechanism, from spontaneous internal stimuli such as action potentials, from a simple "leakage" phenomenon, or...
from a combination of alternatives. Although in the absence of relevant electrophysiological data it is impossible to distinguish among these possibilities, the data do show that the majority of the basal neurotransmitter release is calcium-dependent (Table 1). This should rule out "leakage" via a nonspecific mechanism. Because neurotransmitter release is inhibited to greater than 60% by cobalt ions, it can be concluded that the basal release represents a physiological process analogous to evoked release but less sensitive to calcium deprivation.

Cultures of PC12 cells not exposed to NGF contain 1.5 nmol of ACh and 20 nmol of catecholamines per mg of protein (1, 2, 11) and dense-core vesicles between 40 and 350 nm in diameter (1). No 30- to 50-nm "cholinergic-like" clear vesicles have been observed (F. G. Klier and D. Schubert, unpublished data). When the size of the vesicles was plotted against frequency, a wide distribution was obtained. Reserpine treatment eliminated both the larger size class of vesicles and the stored catecholamines, suggesting that the larger vesicles contain catecholamines. The equilibrium density in a sucrose gradient for the catecholamine-containing vesicles of PC12 is approximately 1.2 M, which is in agreement with the density of catecholamine storage vesicles in bovine splenic sympathetic nerves (12). The particles containing ACh sediment to approximately 1 M sucrose, well above the density of 0.4 M where brain cholinergic vesicles collect (5). An additional experiment independently confirmed the uniqueness of the catecholamine and ACh storage sites. When the catecholamines were depleted by reserpine, the synthesis and storage of ACh was only slightly reduced (Fig. 3). In a catecholamine-depleted culture, ACh is released normally by 50 mM potassium, as are DBH and CGA. Although the release of these proteins is normally associated with catecholamine secretion, adrenal chromaffin cells depleted of catecholamines can still be induced to secrete chromogranins (13). Because reserpine reduces the vesicular storage of DBH and CGA by 80% in PC12 cells, it follows that the vesicle storage and secretion of these proteins are not necessarily coupled.

There are three alternatives that could explain the relationship between the 100-nm dense-core vesicles and ACh storage. (i) This vesicle stores ACh. (ii) The 100-nm vesicle is a precursor to the larger catecholamine-containing vesicle but contains little or no catecholamine itself. (iii) The dense-core vesicles remaining after reserpine depletion are unrelated to neurotransmitter metabolism but perhaps are a result of pinocytic uptake processes. For the last two possibilities to be valid, an alternative storage site or particulate structure is required, because the majority of the ACh does sediment to 1 M sucrose. Trapping of soluble ACh into membranous sacs formed during homogenization was ruled out, and very few closed membrane structures were seen in the gradient fractions containing ACh from reserpine treated cells (Fig. 6). This leaves only the possibilities that a particle present in the cell and in the 1 M sucrose gradient fraction is not visualized by the electron microscopic techniques used, or that ACh is in fact retained within the 100-nm dense-core vesicle. Because the membrane fixation and staining was good in these preparations, we consider the former alternative unlikely. A small fraction of the vesicles may, however, be reserpine-insensitive precursors to catecholamine-containing granules, because approximately 20% DBH and CGA is found in the gradient fractions containing ACh after reserpine treatment relative to control cultures.

If it is established in other undifferentiated nerve cell preparations that ACh is stored in dense-core vesicles and spontaneously released, what is the physiological significance of these phenomena, particularly with reference to early stages of synapse formation? Synapse formation in tissue culture, as defined by the detection of mepps, can be found in times as short as 22 min after mixing two cell types (14). With respect to PC12, synapses on skeletal muscle have been detected within 1 hr after mixing the cells in the absence of NGF (Y. Kidokoro, personal communication), and spontaneous excitatory postsynaptic potentials are found in most PC12 cells that are in contact in clonal culture (A. Ritchie, personal communication). These results show that undifferentiated PC12 cells, in the absence of NGF, have the cellular machinery required to store and release ACh in the apparently quantal fashion necessary to generate mepps in the "postsynaptic" cell. Because the data also suggest that ACh is stored in dense-core vesicles, it follows that similar vesicles may be involved in the storage and possibly the release of ACh in the early stages of synapse formation. Dense-core vesicles of variable size and unknown function are found in numerous nonadrenergic nerves such as sensory fibers (15), motor nerve terminals (16), and autonomic preganglionic fibers (17). In addition, dense-core vesicles are numerous in growing and/or regenerating peripheral nerve fibers (15, 18) and are invariably associated with newly formed nerve-muscle synapses in cell culture (19, 20). Finally, if nerves are continually releasing neurotransmitter by an exocytosis mechanism, it is possible that mepp-like phenomena could be detected on any region of nerve-muscle or nerve-nerve contact that contains a sufficiently high density of the appropriate receptor. Thus, if the growth cone is releasing transmitter as it initially explores the postsynaptic cell, mepps may be detected and the event scored as a synapse. Further studies are required to examine the relationship between events initiated by the basal neurotransmitter release mechanisms and those used at synapses.

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