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Evoked Neuronal Secretion of False Transmitters

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Summary

The ability of differentiated neurons to secrete false transmitters in response to depolarization was examined by loading exogenous transmitters into the neuronal cytoplasm with a whole-cell recording pipette. We found that within minutes following loading of exogenous glutamate into the cytoplasm of cholinergic Xenopus spinal neurons, depolarization-evoked glutamate secretion could be detected by an acutely dissociated hippocampal pyramidal neuron manipulated into contact with the spinal neuron. Similarly, when ACh was loaded into a glutamatergic hippocampal neuron, evoked ACh secretion could be detected by a myocyte. The evoked secretion of the false transmitter was Ca^{2+} dependent and appeared to be vesicular in nature. These results suggest that differentiated neurons are capable of packaging and secreting multiple nonpeptide transmitters, provided that sufficient concentrations of the transmitters are available in the cytoplasm.

Introduction

Synaptic transmission between neurons depends on the synthesis, packaging, and regulated secretion of neurotransmitters from presynaptic nerve terminals (Katz, 1969; Kandel et al., 1991). In general, each differentiated neuron utilizes one type of traditional nonpeptide transmitter together with one or more neuropeptides (Campbell et al., 1982; Jan and Jan, 1983; Hökfelt et al., 1984). In sympathetic neurons, there is substantial evidence for plasticity in the choice of transmitters, and the developmental mechanism that determines the transmitter phenotype is beginning to be understood (Patterson and Chun, 1974; Landis, 1990). Two nonpeptide transmitters, acetylcholine (ACh) and norepinephrine, can be released from both neonatal and adult sympathetic ganglionic neurons (Furshpan et al., 1976, 1986; Potter et al., 1986). However, it is not clear whether the use of a particular transmitter is determined primarily by the availability of the transmitter in the neuronal cytoplasm or whether it is also dependent on the expression of specific proteins involved in transmitter packaging. In the present study, we found that within minutes following loading of exogenous glutamate into the cytoplasm of cholinergic spinal neurons, depolarization-evoked glutamate secretion could be detected by an acutely dissociated hippocampal pyramidal neuron in contact with the spinal neuron. Similarly, when ACh was loaded into a glutamatergic hippocampal neuron, evoked ACh secretion could be detected by a myocyte. In both cases, we found efficient evoked secretion of exogenous false transmitters in a manner consistent with Ca^{2+}-dependent vesicular exocytosis. These results suggest that differentiated neurons are capable of packaging and secreting multiple nonpeptide transmitters. Provision of a sufficient concentration of a new transmitter in the cytoplasm appears to be a critical determinant in the establishment of functional synaptic transmission using the new transmitter.

Results

Secretion of Glutamate from Cholinergic Neurons

Cholinergic spinal neurons in 1-day-old Xenopus nerve–muscle cultures were examined for their capability to secrete glutamate (Figure 1a). Prior to each experiment, the culture was screened for cholinergic neurons by manipulating a whole cell–clamped myocyte into contact with the growth cone of the cocultured neurons. Neurons that elicited spontaneous and evoked synaptic currents in the myocyte (Evers et al., 1989) were considered to be cholinergic and were subsequently used for experiments. For studying glutamate secretion, glutamate was loaded into the cytoplasm of the cholinergic neuron at the soma by a whole-cell recording pipette containing a high concentration of glutamate. An acutely dissociated guinea pig hippocampal CA1 pyramidal neuron (Kay and Wong, 1986) was then manipulated into contact with the growth cone of the Xenopus spinal neuron (Figure 1b). Within a few minutes after the onset of glutamate loading, step depolarizations applied to the spinal neuron frequently produced inward currents resembling excitatory postsynaptic currents (EPCs) in the hippocampal neuron (Figure 1d). These currents were totally abolished by bath application of CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) (20 μM; n = 5 cells), an antagonist of kainate/AMPA (amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors. Subsequent tests of ACh secretion by manipulating a myocyte into contact with the same growth cone (Figure 1c) in the presence of CNQX showed that the same depolarization pulses were capable of inducing EPCs in the myocyte (Figure 1e). The latter currents were abolished by bath application of d-tubocurarine (0.5 mM; n = 5), a specific antagonist of nicotinic ACh channels. In similar experiments, we found that (±)-2-amino-5-phosphonopentanoic acid (25 μM), an N-methyl-D-aspartate (NMDA) receptor antagonist, had no effect on the EPCs observed in the hippocampal neurons (n = 4). Thus, cholinergic neurons were capable of depolarization-evoked secretion of glutamate, and activation of kainate/AMPA receptors in the hippocampal neurons was responsible for the observed currents. The lack of NMDA sensitivity in the

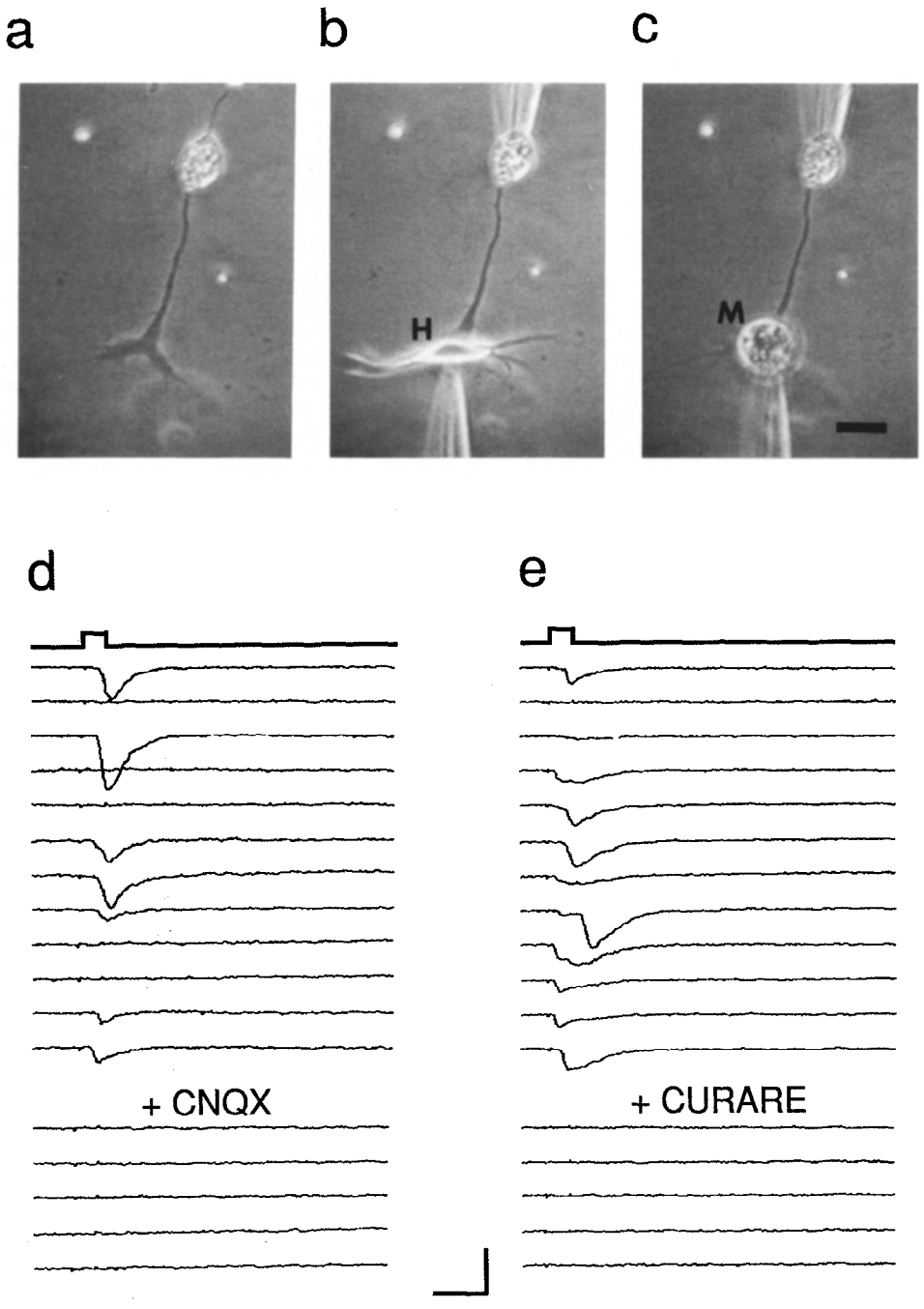

Figure 1. Glutamate and ACh Secretion from Cholinergic Spinal Neurons

(a) Phase-contrast microscopic image of an isolated spinal neuron in a 1-day-old Xenopus nerve-muscle culture.

(b) An acutely dissociated guinea pig hippocampal pyramidal neuron (H) was whole-cell clamped and manipulated into contact with the growth cone of the Xenopus spinal neuron to detect glutamate secretion.

(c) A spherical Xenopus myocyte (M) was manipulated into contact with the growth cone of the same spinal neuron to detect ACh secretion after the hippocampal neuron had been removed. Scale bar, 30 μm.

(d and e) Samples of depolarization-evoked EPCs detected by the hippocampal neuron and the myocyte, respectively. The spinal neuron was whole-cell clamped at −60 mV with a pipette containing 50 mM glutamate. Approximately 10 min after a hippocampal neuron (or a myocyte) was manipulated into contact with the growth cone of the Xenopus neuron, step depolarizations of +70 mV (5 ms duration; shown in the top trace) at a frequency of 0.5 Hz were used to trigger the transmitter secretion. Traces of consecutively recorded membrane currents at a hippocampal neuron (d) or a myocyte (e) are shown (filtered at 1 and 5 kHz), respectively, before and after bath application of receptor antagonists CNQX (20 μM) and d-tubocurarine (0.5 mM). The hippocampal neuron and the myocyte were voltage clamped at −90 and −70 mV, respectively. Scales are 50 pA for 10 ms in (d), and 500 pA for 10 ms in (e).

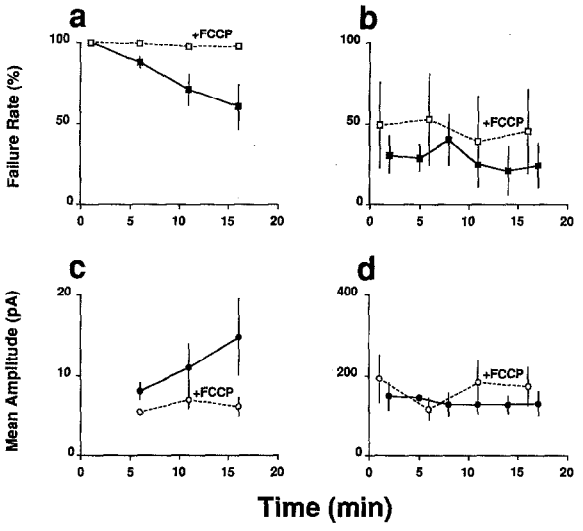

Figure 2. Properties of Glutamate and ACh Secretion by Xenopus Spinal Neurons

(a and b) Percentage of failure in eliciting EPCs in the hippocampal neuron and the myocyte, respectively. In (a), the hippocampal neuron was voltage clamped at −90 mV and the spinal neuron was loaded with 50 mM of glutamate through the whole-cell pipette at the soma. Time 0 represents the onset of glutamate loading. Step depolarizations of +70 mV (duration, 5 ms) were applied to the spinal neuron at the soma at a frequency of 0.5 Hz. Data depict average values from 13 separate experiments, and each point represents the mean value obtained over a 1 min period. The dashed line connects data from a parallel set of 20 experiments in which 50 μM FCCP, an ionophore that dissipates the proton gradient across the vesicle membrane, was added in the pipette solution in addition to 50 mM glutamate. Error bars refer to SEM. In (b), similar experiments were performed on a separate group of spinal neurons for evoked ACh secretion, using myocytes as detector cells. Time 0 represents onset of neuron-myocyte contact. Data were from three separate experiments.

(c and d) Mean amplitudes of EPCs for the same set of experiments as that shown in (a) and (b). Each point represents the mean value of all detectable EPCs (amplitude, >5 pA).

hippocampal neurons is presumably due to the disruption of NMDA receptors during the acute dissociation procedure (see Experimental Procedures). In the absence of glutamate loading, no EPC was observed in the hippocampal neuron in all cases (n = 20), suggesting that there was no detectable evoked secretion of endogenous glutamate from these cholinergic neurons.

Time Course and Concentration Dependence of Glutamate Secretion

The appearance of evoked glutamate secretion was compared with that of the evoked ACh secretion from these Xenopus spinal neurons. After the onset of glutamate loading (50 mM), the rate of failure in eliciting glutamate secretion decreased, whereas the mean amplitude of the detectable EPCs (amplitude, >5 pA) increased with the loading time (Figures 2a and 2c). In contrast, EPCs induced by evoked ACh secretion from these neurons showed no obvious change in either the rate of failure or mean amplitude (Figures 2b and 2d). This difference may be accounted for by the time required for the diffusion of glutamate from the soma to presynaptic nerve terminals (Popov and Poo, 1992) and for packaging of glutamate prior to depolarization-dependent exocytosis. The efficiency of excitation-secretion coupling for glutamate, after 10 min of loading, was comparable to that for ACh. This is suggested by the same delay in the appearance of EPCs following the onset of the 5 ms step depolarizations, namely, 3.7 ± 0.3 ms (SEM; n = 5) and 3.5 ± 0.4 ms (SEM; n = 3) for glutamate and ACh secretion, respectively.

In a series of experiments, the concentration of glutamate in the filling solution for the whole-cell recording pipette was varied from 15 to 170 mM. The failure rate in eliciting glutamate secretion decreased and the mean amplitude of the detectable EPCs increased with increasing concentration of glutamate (Figure 3). Detectable evoked glutamate secretion was observed in the majority of cells (17 of 20) at 50 mM and occasionally (5 of 16) at 15 mM. Since EPCs with amplitudes below the noise level were not detected, the higher failure rate after loading a lower concentration of glutamate could result in part from a reduced amplitude of EPCs.

Comparisons of Glutamate and ACh Secretion

We have further characterized the properties of glutamate and ACh secretion from these cholinergic spinal neurons. We first examined the Ca^{2+} dependence of the secretion. As shown in Figure 4, the failure rates in eliciting glutamate and ACh secretion from the neuron were both increased after addition of 10 mM of Mg^{2+} in the extracellular medium, and the secretion was completely abolished by the addition of 10 mM of Co^{2+}. These two treatments are known, respectively, to reduce or to prevent depolarization-induced Ca^{2+} influx into these Xenopus neurons (Sun and Poo, 1987). Thus, secretion of glutamate and ACh from these neurons depends on mechanisms that have similar requirements for Ca^{2+} influx.

By manipulating the detector cell into contact with different regions of the spinal neuron, we also examined the regional differences in the capability of transmitter secretion for both endogenous and false transmitters. The site of evoked glutamate secretion appeared to be preferentially localized to the growth cone. When glutamate secretion was monitored by the hippocampal neuron at the growth cone, along the length of the neurite, and at the soma of the spinal neuron, evoked glutamate secretion was detected in 85%, 20%, and 5%, respectively, of all the neurons examined (n = 20). Evoked ACh secretion from these cholinergic neurons detected by the myocyte is similarly localized to the growth cone. The frequencies of detecting ACh secretion at the growth cone, along the neurite, and at the soma are 100%, 27%, and 7%, respectively (n = 15).

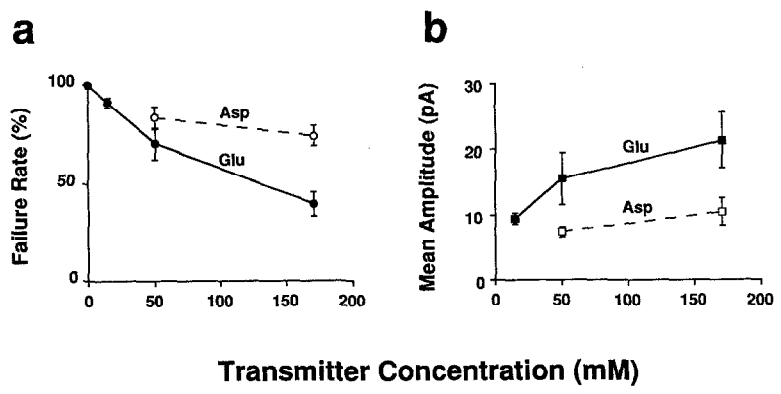

Figure 3. Evoked Transmitter Secretion from Xenopus Spinal Neurons after Loading with Different Concentrations of Glutamate or Aspartate

The mean failure rate and the mean amplitude of detectable EPCs (amplitude, >5 pA) as detected by hippocampal neurons 10 min after glutamate loading were plotted against the concentration of glutamate (Glu) or aspartate (Asp) in the loading pipette. Each data point represents mean value (± SEM) from at least 16 experiments.

Finally, we have compared the effect of hypertonic solution, which is known to induce an increase in the frequency of spontaneous transmitter release at many synapses (Fatt and Katz, 1952; Bekkers and Stevens, 1989), presumably as a result of enhanced exocytosis of synaptic vesicles. Local perfusion of hypertonic solution (0.5 M sucrose in the bath solution) was applied to the cholinergic neurons 10 min after the onset of glutamate loading. An increased frequency of events resembling miniature EPCs was observed in the detector hippocampal cells immediately after the perfusion (Figure 5a), reflecting increased spontaneous quantal glutamate secretion. In parallel experiments, similar perfusion of hypertonic solution induced an increased frequency of spontaneous quantal ACh secretion, as detected by a myocyte (Figure 5b). This indicates that the spontaneous glutamate and ACh secretion depends on exocytotic mechanisms that are similarly enhanced by hypertonic treatment. We noted that in the absence of hypertonic solution, spontaneous glutamate secretion was rarely observed, although spontaneous ACh secretion occurred frequently. Previous studies have shown that specific myocyte contact with these spinal neurons results in an induction of spontaneous ACh secretion (Xie and Poo, 1986). The lack of spontaneous glutamate secretion may be due to the inability of guinea pig hippocampal neurons to induce spontaneous secretion from Xenopus spinal neurons.

Sensitivity of Glutamate Secretion to Carboxyl Cyanide p-Trifluoromethoxy-Phenylhydrazone

The observation of spontaneous quantal glutamate secretion after the hypertonic treatment also suggests that exogenous glutamate molecules are packaged into cytoplasmic vesicles prior to secretion. This idea was further supported by the finding that cytoplasmic loading of glutamate together with carboxyl cyanide

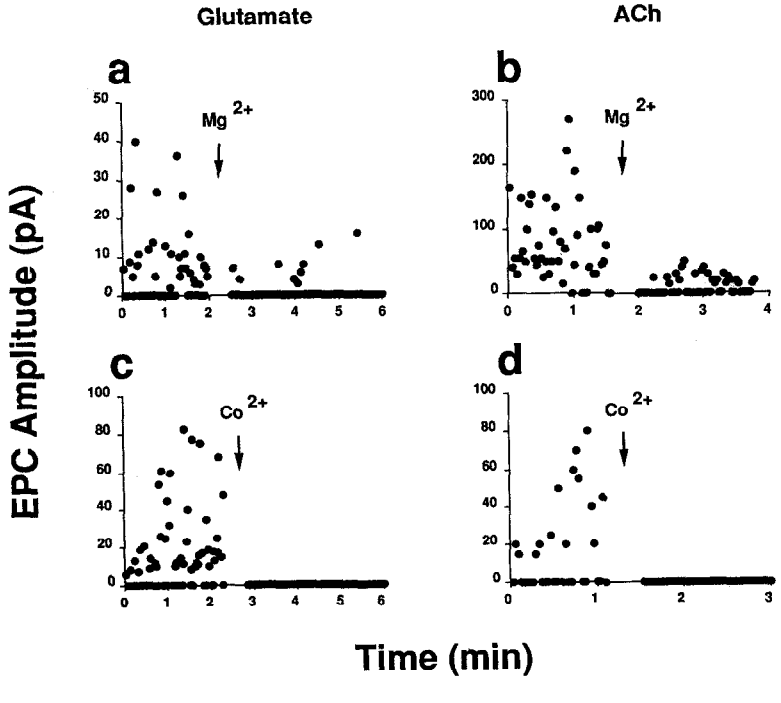

Figure 4. Ca^{2+} Dependence of Glutamate and ACh Secretion from the Cholinergic Xenopus Spinal Neurons

The effect of extracellular Mg^{2+} and Co^{2+} was examined for glutamate secretion (a and c) from Xenopus spinal neurons loaded with glutamate using a hippocampal neuron as a detector cell and for ACh secretion (b and d) as detected by a myocyte. The amplitude of EPCs observed at various times before and after bath application (at time marked by the arrow) of 10 mM Mg^{2+} or 10 mM Co^{2+} were plotted. Data from one experiment were shown for each case.

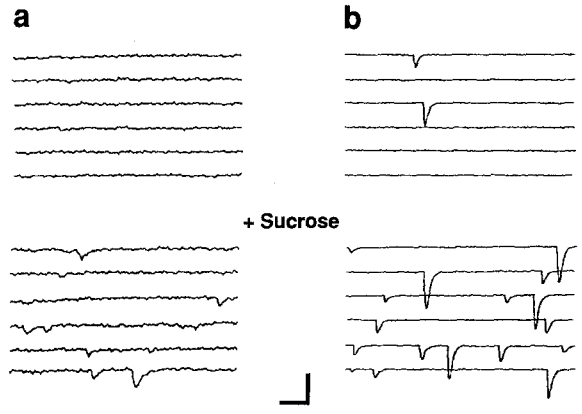

Figure 5. Increase in Spontaneous Secretion of Glutamate and ACh Following the Treatment with Hypertonic Solution as Shown by the Marked Increase in the Frequency of Miniature EPCs

(a) Glutamate. (b) ACh. Oscilloscope traces are membrane currents recorded in the detector cells before (upper traces) and after (lower traces) perfusion of cholinergic spinal neurons with recording medium containing 0.5 M sucrose. The neuron was loaded with 50 mM glutamate, and glutamate or ACh secretion was detected at the growth cone of the neuron in the same manner as that described in Figure 1. The data were from two different spinal neurons. Scales are 20 pA for 20 ms in (a) and 200 pA for 20 ms in (b).

p-trifluoromethoxy-phenylhydrazone (FCCP) (50 μM), an ionophore that dissipates the proton gradient across the vesicle membrane (Maycox et al., 1988), significantly inhibited evoked glutamate secretion (see Figures 2a and 2c). This drug treatment apparently affected the vesicular packaging of glutamate but not other components of the secretion machinery, since the same loading of FCCP did not produce significant changes in the evoked ACh secretion over the duration of these experiments (see Figures 2b and 2d). This is consistent with the previous finding that FCCP inhibits glutamate uptake into glutamatergic synaptic vesicles (Maycox et al., 1988). The lack of FCCP effect on ACh secretion from these cholinergic neurons also suggests that prepackaged ACh quanta were not affected by the drug over the duration of these experiments.

Effect of Loading Aspartate

Aspartate has been proposed as another excitatory amino acid transmitter used in the central nervous system (Bradford, 1986). We have loaded aspartate into the cholinergic Xenopus spinal neurons and used the hippocampal neurons to monitor evoked transmitter secretion. Similar EPCs as those found after glutamate loading were observed. However, aspartate loading resulted in EPCs with higher failure rates and smaller amplitudes than those observed after loading of similar concentrations of glutamate (see Figure 3). These EPCs observed after aspartate loading were totally abolished by bath application of CNQX, an antagonist for kainate/AMPA receptors. Kainate/AMPA receptors, however, are presumably not activated by aspartate. Furthermore, pulse application of aspartate (at 1 mM) at the surface did not evoke any response in these hippocampal neurons, while similar glutamate application induced substantial inward currents. Thus, the observed EPCs appear to result from secretion of glutamate that was converted from aspartate in the cytoplasm of these cholinergic neurons, presumably through the rapid action of aspartate aminotransferase (Bradford, 1986).

Secretion of ACh from Hippocampal Pyramidal Neurons

In a separate series of experiments, we also examined the ability of acutely dissociated hippocampal CA1 pyramidal neurons to secrete ACh. Since the acute dissociation procedure had disrupted the processes of the hippocampal neuron, we monitored ACh secretion from the soma. The hippocampal neuron was loaded with 30 mM ACh through a whole-cell recording pipette and manipulated into contact with a whole cell-clamped Xenopus myocyte, which served for ACh detection (Figure 6a). Within 5–10 min following the onset of ACh loading, depolarization-evoked EPC-like currents were observed in the myocyte (Figure 6b). These currents were abolished by bath application of d-tubocurarine (0.5 mM), indicating that they were induced by ACh secretion. No EPC was observed in the myocyte for all hippocampal neurons not loaded with ACh ($n = 10$), suggesting that there is no endogenous ACh secretion from these neurons. In these experiments, stable contact was consistently made only between the soma of the dissociated hippocampal neuron and the myocyte. The secretion is thus likely to be somatic, although contribution from remnants of axonal processes cannot be excluded (see Discussion).

As shown in Figures 7a and 7b, EPCs resulting from ACh secretion by hippocampal neurons showed increasing mean amplitude and decreasing failure rate during the first 10–20 min after the onset of ACh loading, similar to that of glutamate secretion from cholinergic Xenopus neurons. Since the cytoplasmic ACh concentration in cholinergic nerve terminals was estimated to be about 30 mM (Miledi et al., 1977; Kriebel et al., 1978; Israel et al., 1979), we loaded hippocampal neurons with 3–30 mM of ACh. With increasing concentration of ACh, the mean amplitude of EPCs detected in the myocytes increased and the mean failure rate of evoking ACh secretion decreased (Figures 7c and 7d). Furthermore, cytoplasmic loading of FCCP (50 μM) together with ACh significantly inhibited the evoked ACh secretion from the hippocampal neuron (Figures 7a and 7b), suggesting that the packaging of ACh in these neurons also requires the proton gradient across the vesicle membrane. The delay in the appearance of EPCs in the myocytes following the onset of step depolarizations applied to hippocampal neurons was 2.3 ± 0.5 ms (SEM; $n = 6$), suggesting rather efficient excitation–secretion coupling for

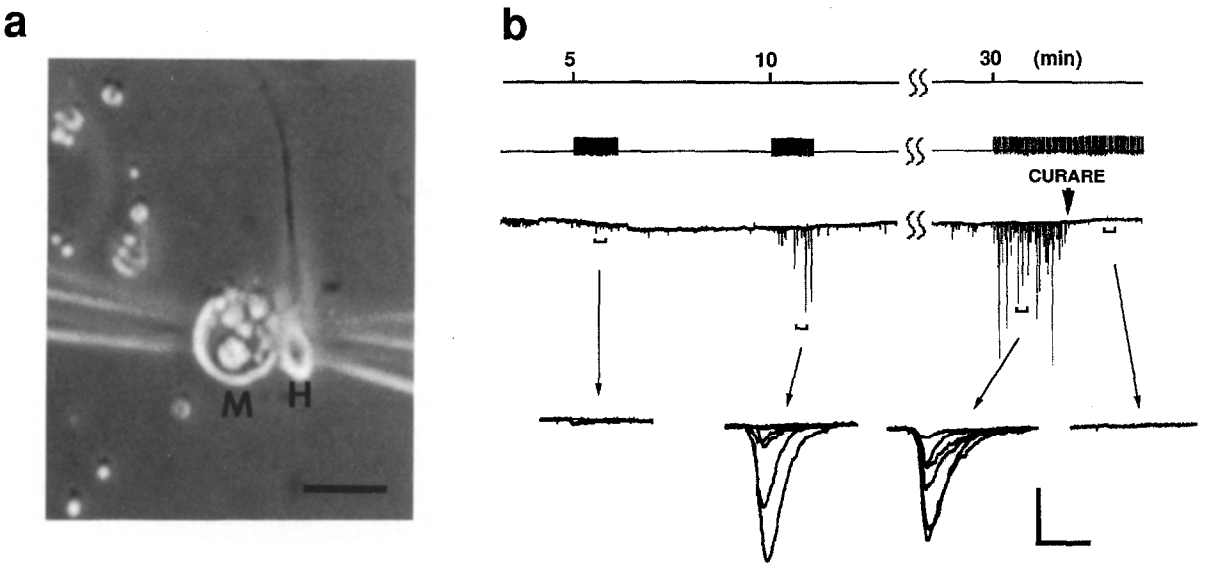

Figure 6. Secretion of ACh from Acutely Dissociated Guinea Pig Hippocampal Neurons after Cytoplasmic Loading with ACh

(a) Phase-contrast image of an acutely dissociated hippocampal pyramidal neuron (H) manipulated into contact with a whole cell-clamped myocyte (M) from 1-day-old Xenopus culture. The hippocampal neuron was whole-cell clamped at −65 mV with a pipette containing 30 mM ACh. Scale bar, 30 μm.

(b) The upper continuous trace depicts pattern of depolarization pulses (+100 mV, 10 ms, 0.5 Hz) applied to the hippocampal neuron to elicit ACh secretion. The lower continuous trace depicts membrane currents in a voltage-clamped myocyte (Vc = −70 mV), after a hippocampal neuron loaded with 30 mM ACh was manipulated into contact. Time 0 refers to the onset of ACh loading. Samples of EPCs recorded in the myocyte at various times are shown below at a higher time resolution. The arrow marks the time of bath application of d-tubocurarine (0.5 mM). Scales are 300 pA for 20 ms.

ACh. The hippocampal neurons used in these experiments are known to be glutamatergic (Cooper et al., 1991). The present results indicate that adult glutamatergic neurons are capable of efficient evoked secretion of exogenous ACh soon after cytoplasmic loading.

Discussion

The main finding of the present work is the efficient evoked secretion of exogenous false transmitters by cholinergic and glutamatergic neurons within minutes following the onset of cytoplasmic loading. Previous work has demonstrated the capacity of spinal motor neurons and hippocampal neurons to secrete the false transmitters acetylmonoethylcholine and D-glutamate, respectively (Colquhoun et al., 1977; Pan et al., 1993). These false transmitters are structural analogs of the endogenous transmitters used at the synapses. In the present study, we have demonstrated that the cholinergic and glutamatergic neurons can effectively secrete false transmitters that are not structurally related to their endogenous transmitters. This indicates that the differentiated neurons may be capable of using multiple nonpeptide transmitters available in the neuronal cytoplasm for synaptic transmission. The secretion of two endogenous nonpeptide transmitters has been shown in sympathetic neurons (Furshpan et al., 1976, 1986; Potter et al., 1986) and serotonergic neurons (Johnson, 1994). In these earlier studies, it is not clear what specific molecular and cellular components are required to endow the neuron with the capability of secreting multiple nonpeptide transmitters. The present study provides a direct demonstration that provision of a new transmitter in the cytoplasm is sufficient for the evoked secretion of that transmitter. Additional components in the neuron may facilitate the processes of transmitter packaging and secretion.

The average sizes of evoked current responses observed in the detector cells for false transmitter secretion are smaller than those observed at several glutamatergic or cholinergic synapses (Bekkers and Stevens, 1990; Manabe et al., 1992; Evers et al., 1989). This is reasonable in view of the limited glutamate and ACh sensitivity of hippocampal soma and myocyte, respectively, as compared with the expected high density of receptors at subsynaptic membranes. Limited surface area of contact between the secreting and detecting cells may also reduce the observed current responses. Depending upon the packaging mechanism (see below), the concentration of the false transmitters in the vesicles could be lower than that of endogenous transmitters. Nevertheless, these evoked responses generated by false transmitters are quite similar to those observed at natural synapses during the initial phase of synaptogenesis (Evers et al., 1989); they are sufficient to achieve functional synaptic transmission, since the released transmitters produce clearly detectable signals in an adjacent cell.

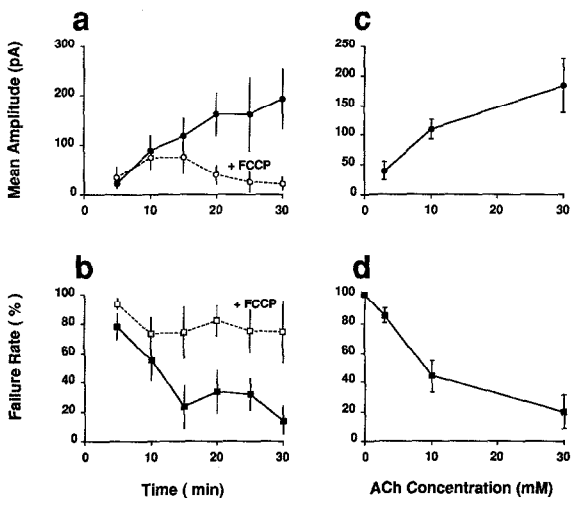

Figure 7. Secretion of Exogenous ACh from Hippocampal Neurons

(a and b) Time course of ACh secretion detected in the myocyte after loading the hippocampal neuron with 30 mM ACh. Time 0 refers to the onset of ACh loading. The mean amplitude of detectable EPCs (amplitude > 10 pA) and the mean failure rate were plotted against time after ACh loading. Data connected by solid lines were obtained from five separate loading experiments, and the points represent mean value (± SEM). Data connected by dashed lines were obtained from a parallel set of five experiments, in which the loading pipette contained 50 μM FCCP and 30 mM ACh.

(c and d) The dependence of ACh secretion on the concentration of ACh in the loading pipette. The mean failure rate of evoked ACh secretion and the mean amplitude of detected EPCs 30 min after the onset of ACh loading were plotted against the ACh concentration. Data points represent mean value (± SEM) from five experiments.

There are several lines of evidence suggesting that the false transmitters are secreted through a vesicular exocytotic pathway similar to that for endogenous transmitter secretion. The evoked secretion of false transmitter depends on the influx of Ca^{2+}, with a latency in excitation-secretion coupling similar to that of endogenous transmitter. The spontaneous quantal secretion, which is generally accepted as vesicular in mechanism, was elevated in frequency by hypertonic treatment for both the exogenous and endogenous transmitters. That the false transmitters were packaged into cytoplasmic vesicles prior to secretion is further supported by the findings on the effect of FCCP. A proton gradient across the vesicular membrane is known to be required for vesicular accumulation of transmitters (Maycox et al., 1988, 1990; Anderson et al., 1982). In the present study, disruption of the proton gradient markedly inhibited the evoked secretion of false transmitters. The characteristic time for the appearance of evoked false transmitter secretion was in the range of 5–10 min. This is also comparable to the time course observed for the uptake of ACh and L-glutamate by purified Torpedo and brain synaptic vesicles, respectively (Anderson et al., 1982; Maycox et al., 1988). Taken together, these results suggest that the false transmitters are packaged into similar or the same vesicles as those used for endogenous transmitters. A number of neurotoxins are known to inhibit the exocytosis of synaptic vesicles (Simpson, 1989; Schiavo et al., 1992; Blasi et al., 1993). Future studies of their effect on the secretion of false transmitters will verify whether false transmitter secretion utilizes the same vesicular pathway.

Transmitter uptake into synaptic vesicles is thought to be mediated by specific vesicular transporter proteins (Edwards, 1992; Kelly, 1993). The important question to address is whether packaging of false transmitters is due to nonspecific permeation and vesicular trapping of the false transmitter or to the action of specific transporter proteins. The latter would require the existence of multiple transporters within differentiated neurons, evidence of which is lacking at present. A nonspecific accumulation of false transmitters appears more likely to account for our results, in view of the recent findings that quantal ACh secretion can be observed in myocytes (Dan and Poo, 1992) and fibroblasts (Alder et al., 1993) after cytoplasmic loading of ACh. Nonneuronal cells are unlikely to possess synaptic vesicle transporters, although one cannot exclude the possibility that homologous proteins serving similar transport functions may exist in these cells. Nonspecific vesicular accumulation of ACh has also been demonstrated previously for purified synaptic vesicles from Torpedo electric organ (Carpenter et al., 1980). Regardless of the packaging mechanisms, nonspecific or transporter mediated, our results of FCCP experiments suggest that vesicular accumulation of false transmitters requires a proton gradient across the vesicle membrane. The requirement for a proton gradient has also been shown for transporter-mediated uptake of glutamate and ACh into synaptic vesicles (Maycox et al., 1988, 1990; Anderson et al., 1982).

The hippocampal CA1 pyramidal neurons used in these experiments are known to be glutamatergic in nature (Cooper et al., 1991). The finding of efficient evoked ACh secretion from the soma of these neurons suggests that the transmitter secretion machinery was present in the soma, perhaps through retention of remnants of axonal processes after acute dissociation. Alternatively, ACh loaded into the cytoplasm may utilize the exocytosis machinery in the recycling or constitutive secretion pathway located in the soma, which has similar Ca^{2+}-dependent properties as that of the synaptic vesicle exocytosis. Ca^{2+} dependence in the quantal secretion of exogenous ACh through constitutive or recycling exocytotic pathway has recently been demonstrated in myocytes (Dan and Poo, 1992) and fibroblasts (Alder et al., 1993, Soc. Neurosci., abstract; Popov et al., submitted).

The average concentration of glutamate in the mammalian brain is estimated to be about 10 mM (Maycox, et al., 1990; Ottersen and Storm-Mathisen, 1984). The cytoplasmic glutamate concentration in glutamatergic neurons is uncertain; however, it is likely to be much

higher than 10 mM. In nonglutamatergic neurons, a reasonable guess would be on the order of a few millimolars. Our finding that detectable glutamate secretion from cholinergic neurons was observed after loading with 15 mM of glutamate is of particular interest. First, the level of exogenous false transmitter we loaded in the neuron was likely to be within the physiological range. Second, in nonglutamatergic neurons, the low concentration of cytoplasmic glutamate might not support physiologically detectable evoked secretion. On the other hand, a lower level of evoked secretion of endogenous glutamate, while undetectable by the present method, may nevertheless exist naturally. There is indeed biochemical evidence that suggests corelease of glutamate and ACh from some cholinergic nerve terminals (Parsons et al., 1993; Docherty et al., 1987; Vyas and Bradford, 1987; Saji and Miura, 1991). This ubiquitous form of evoked glutamate secretion may exert important regulatory actions in the nervous system.

In conclusion, while the mechanism for the packaging of false transmitters is uncertain, the present results clearly demonstrate the capacity of differentiated neurons for utilizing exogenous false transmitters. The rapidity of the packaging of false transmitters and the efficiency of their evoked secretion underscore the dynamic and plastic nature of the neuronal secretion mechanism.

Experimental Procedures

Cell Preparations

The Xenopus neurons and myocytes were prepared by previously reported methods (Spitzer and Lamborghini, 1976; Tabti and Poo, 1991) and used for experiments after 1 day of culturing at room temperature (20°C–22°C). The culture medium consisted of 50% (v/v) of Ringer's solution (115 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, 10 mM HEPES [pH 7.6]), 49% of Leibovitz medium (L15; GIBCO), and 1% fetal bovine serum (GIBCO). Acutely dissociated hippocampal neurons were prepared by the method of Kay and Wong (1986). In brief, hippocampi from 1-month-old guinea pigs were dissected and cut into 600 μm thick slices. CA1 region of the slices were dissected and dissociated in PIPES solution containing trypsin (0.8 mg/ml) for 2 hr at 30°C in the presence of saturated O_2 and then washed with fresh PIPES solution at room temperature. Neurons were obtained from washed pieces of tissues by gentle trituration with fire-polished pipettes and used for up to 8 hr after the trypsin treatment. After this treatment, the neuron retained glutamate sensitivity, although NMDA receptors were largely disrupted. For measuring glutamate secretion, pieces of glass coverslips plated with Xenopus spinal neurons and myotomal myocytes were transferred into a recording chamber containing dissociated hippocampal neurons. For detecting ACh secretion, the myocyte was detached from the culture substratum by a micropipette, and whole-cell recording was achieved on the detached cell before it was manipulated into contact with the growth cone of the Xenopus spinal neuron.

Electrophysiology

The membrane currents in the detector cell (hippocampal neuron or myocyte) were monitored by gigaohm-seal, whole-cell recording method (Hamill et al., 1981; Young and Poo, 1983), using a patch-clamp amplifier (Axopatch-1D). The recorded currents were filtered at 1–5 kHz and stored in a videotape recorder for later playback onto a storage oscilloscope (Textronic 5113) or an oscillographic recorder (Gould RS3200) and for analysis by a microcomputer. Internal solution in the recording pipette for myocytes contained 150 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$, and 10 HEPES buffer (pH 7.2), and internal solution for hippocampal neurons contained 120 mM KF, 11 mM BAPTA, 10 mM HEPES, 5 mM ATP, 0.5 mM GTP, and 0.1 mM leupeptin (pH 7.2). All recordings were made at room temperature in solution containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 24 mM D-glucose, 10 mM HEPES (pH 7.4). For loading 50 mM glutamate into the cholinergic neurons, the filling solution for the whole-cell recording pipette contained 1 mM $MgCl_2$, 1 mM NaCl, 50 mM K-glutamate, 120 mM K-gluconate, 5 mM ATP, 0.5 mM GTP, and 10 mM HEPES (pH 7.2). For different concentrations of glutamate loading, K-glutamate was interchanged with K-gluconate to achieve the final osmolarity of 310 mOsm. For loading 30 mM ACh into the soma of hippocampal neurons, the whole-cell recording pipette contained 30 mM AChCl, 100 mM KF, 10 mM HEPES, 5 mM ATP, and 0.5 mM GTP (pH 7.2). Adjustment of ACh concentration was done by substituting AChCl with KF, with the final osmolarity of 260 mOsm. Loading of neurons through the whole-cell pipettes is effective. The time course of transmitter loading, as assayed by loading of fluorescent dyes, suggests that the concentration in the soma reached a plateau after 5–10 min. Diffusion of transmitter along the length of neurite, which is at most 100 μm in these experiments, has a characteristic time of about 1 min, assuming a diffusion coefficient for small molecules in the neuronal cytoplasm to be 1×10^{-6} cm^2/s (Popov and Poo, 1992). Thus, we expect that the cytoplasmic concentration of the false transmitters will be similar to that in the whole-cell pipette after 10 min of loading.

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