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Constitutive Secretion of Exogenous Neurotransmitter by Nonneuronal Cells: Implications for Neuronal Secretion

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ABSTRACT Fibroblasts in cell culture were loaded with exogenous neurotransmitter acetylcholine (ACh). ACh secretion from loaded cells was detected by whole-cell patch clamp recordings from *Xenopus* myocytes manipulated into contact with ACh-loaded cells. Two different approaches were used for ACh loading. In the first approach, fibroblasts were incubated in the culture medium containing ACh. Recordings from myocytes revealed fast inward currents that resemble miniature endplate currents found at neuromuscular synapses. The currents observed in recordings from myocytes were due to exocytosis of ACh-containing vesicles. Although exogenous ACh penetrated through the plasma membrane of fibroblasts during incubation and was present in the cytoplasm at detectable levels, cytoplasmic ACh did not contribute to the quantal ACh secretion. In the second approach, exogenous ACh was loaded into the cytoplasm of fibroblasts by microinjection. Under these experimental conditions, fibroblasts also exhibited spontaneous quantal ACh secretion. Analysis of the exocytotic events in fibroblasts following two different protocols of ACh loading revealed that the vesicular compartments responsible for uptake of exogenous ACh are associated with the endocytic recycling pathway. Extrapolation of our results to neuronal cells suggest that in cholinergic neurons, in addition to genuine synaptic vesicles, ACh can be secreted by the vesicles participating in endosomal membrane recycling.

INTRODUCTION

Two forms of exocytosis have been identified in eucaryotic cells. The regulated secretory pathway involves packaging of exported molecules into specialized secretory vesicles, storage of the vesicles near the release sites, and rapid exocytosis in response to an appropriate stimulus. The best-understood example of regulated secretion is neurotransmitter release from the neurons. Accumulation of neurotransmitters in synaptic vesicles at the nerve terminal is mediated by specific transporters. The fusion of synaptic vesicles with the plasma membrane at the nerve terminal is triggered by the rapid elevation of cytoplasmic Ca^{2+} $[Ca^{2+}]_i$ during an action potential (Augustine et al., 1987; Bennett, 1997). The targeting of synaptic vesicles to the release sites and tight excitation-secretion coupling observed at the nerve terminal are mediated by proteins specific to synaptic vesicles (Sudhof, 1995; Calacos and Scheller, 1996; Hanson et al., 1997).

The constitutive (or nonregulated) secretory pathway operates in all cells and is responsible for recycling of plasma membrane components and for secretion of molecules into the extracellular environment. In contrast to synaptic vesicle exocytosis, constitutive vesicular exocytosis occurs at resting $[Ca^{2+}]_i$ levels. Despite different sensitivities to $[Ca^{2+}]_i$, the pathways of synaptic vesicles at the nerve terminal and of endosomal membranes in nonneuronal cells are mechanistically similar. Both are local and do not depend on the Golgi apparatus. Retrieval of synaptic vesicles after neurotransmitter release is believed to occur through the formation of clathrin-coated vesicles, followed by their uncoating, fusion with endosomes, and sorting of synaptic vesicle proteins during budding from endosomes (Calacos and Scheller, 1996). Although the term "constitutive" implies the constant flux of secretory products to the plasma membrane, emerging evidence suggests that various trafficking steps involved in the constitutive secretion pathway may be regulated by calcium (Buys et al., 1984; Beckers and Balch, 1989; Dan and Poo, 1992; Steinhardt et al., 1994; Coorsen et al., 1996; Rodriguez et al., 1997). Moreover, molecular characterization of the secretion machinery components in both neuronal and nonneuronal cells has demonstrated that constitutive and regulated secretion pathways share homolog proteins (Schiavo et al., 1992; Bennett and Scheller, 1993; Sollner et al., 1993). These findings have prompted the suggestion that the transmitter secretion pathway at the nerve terminal has developed through addition of synaptic vesicle-specific proteins to the ubiquitous endosomal membrane recycling pathway. Therefore, the introduction of exogenous neuronal proteins into nonneuronal cells, combined with assays for membrane recycling and exocytosis, may provide information on the functional roles of neuronal proteins in the exocytotic process.

This idea has provided a rationale for the attempts to reconstitute the molecular machinery for neurotransmitter secretion in nonneuronal cells (Cavalli et al., 1991; Alder et al., 1992; Morimoto et al., 1995). One of the functional assays for exocytosis in nonneuronal cells is based on the

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loading of exogenous ACh into nonneuronal cells. Surprisingly, ACh microinjection into the cytoplasm of *Xenopus* myocytes (Dan and Poo, 1992), or frog fibroblasts, in cell culture (Girod et al., 1995) resulted in the accumulation of ACh in the membrane compartments and quantal ACh release, as detected by whole-cell patch clamp recordings from the *Xenopus* myocytes. Moreover, secretion of exogenous ACh from these nonneuronal cells was found to be Ca^{2+}-dependent (Girod et al., 1995). These results suggest that the rudimentary molecular machinery for the vesicular uptake of cytoplasmic ACh and quantal Ca^{2+}-dependent secretion may exist in nonneuronal cells. However, the nature of the vesicles capable of accumulating exogenous ACh, as well as the mechanism of cytoplasmic ACh penetration into the vesicles, remains unclear. The existing evidence suggests that these vesicles may be of lysosomal (Rodriguez et al., 1997), trans-Golgi (Chavez et al., 1996), or endosomal (Miyake and McNeil, 1995) origin.

In the present study we characterized the properties of secretory events in fibroblasts loaded with exogenous ACh by incubation in the ACh-containing culture medium (Morimoto et al., 1995). We demonstrate that after incubation in the ACh-containing medium, exogenous neurotransmitter is taken up by nonspecific fluid-phase endocytosis. Quantal ACh secretion, as revealed by whole-cell *Xenopus* myocytes, reflects exocytosis of vesicles involved in the endosomal membrane recycling pathway. Analysis of the frequency, amplitude, time course, and Ca^{2+} sensitivity of the secretory events revealed a similarity between these events and the previously characterized secretory events following ACh microinjection into the cytoplasm of nonneuronal cells. Our results suggest a close association between constitutive endosomal membrane recycling and spontaneous neurotransmitter secretion in neuronal cells.

MATERIALS AND METHODS

Cell cultures

Amphibian fibroblasts (cell line FT, American Tissue Culture Collection, Rockville, MD) were grown at 25°C and 5% CO_2 environment in MEM (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (GIBCO-BRL, Bethesda, MD), and nonessential amino acids (pH 7.6). Fibroblasts were plated at low density on the acid-washed coverslips and used for experiments 1–5 days after subculturing. Cultured *Xenopus* myocytes were prepared according to previously reported methods (Anderson et al., 1977). Briefly, neural tube and myotomal tissue of 19–24 stage embryos were dissociated in Ca^{2+}-Mg^{2+}-free solution (115 mM NaCl, 2.6 mM KCl, 10 mM HEPES, 0.4 mM EDTA, pH 7.6). Dissociated cells were plated on the surface of plastic petri dishes. The cultures were kept at 20°C in a culture medium consisting of 50% (v/v) saline solution (115 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, 10 mM HEPES [pH 7.6]) and 49% L-15 Leibovitz medium (Gibco BRL) containing 1% fetal bovine serum (Gibco BRL). The myocytes were used for experiments 24–36 h after plating.

Incubation conditions to load endosomal compartments

Fibroblasts were incubated in L15-based *Xenopus* culture medium (pH 7.6) containing 40 mM ACh for various periods of time ranging from 2 to 20 min. Coverslips with plated fibroblasts were extensively washed with fresh culture medium and transferred to the petri dish containing *Xenopus* myocytes. No changes in the morphology of fibroblasts were observed after incubation with ACh. For endocytic loading of radioactive sucrose, fibroblasts were incubated for 10 min in the culture medium containing 50 μCi/ml of ^{14}C-sucrose (ICN Biochemicals, Costa Mesa, CA). After 5 min of extensive washing, the radioactivity released into the culture medium (1 ml) was measured at 5-min intervals using a scintillation counter (Beckman, Carlsbad, CA). All incubations and washings were performed at room temperature. The osmolarity of all solutions used in the experiments was adjusted to 285 mOsm.

Micromanipulation of *Xenopus* myocytes into contact with fibroblasts

Manipulation of *Xenopus* myocytes followed that described previously (Chow and Poo, 1985). Briefly, myocytes were gently detached from the surface of the petri dish by a heat-polished micropipette attached to a hydraulic micromanipulator (Newport, Irvine, CA). The fibroblasts chosen for experiments were free of contact with other cells. The myocytes were transferred in the vicinity of the fibroblast, allowed to reattach to the glass surface, and manipulated into contact with the perinuclear region of the fibroblast. During electrophysiological recordings the myocyte was firmly attached to the surface of the coverslip and was in tight contact with the fibroblast. We found that myocytes plated on the plastic surface were easier to detach from the substrate than those plated on glass coverslips. Therefore, petri dishes were routinely used for myocyte culture preparation.

Electrophysiology

Gigaohm-seal whole-cell recording methods followed those described previously (Hamill et al., 1981). Patch pipettes were fabricated from glass micropipettes (VWR, West Chester, PA) and pulled with a two-step puller (Narishigi, East Meadow, NY). After heat-polishing, the pipette tip diameter was 1.5–2 μm and the resistance was 2–5 MΩ. The intrapipette solution for the whole-cell recording from myocytes contained 140 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). In experiments using ionomycin and ATP (see below), 10 mM BAPTA (Sigma) was added to the intrapipette solution to avoid myocyte damage due to the influx of Ca^{2+}. All recordings were done at room temperature. The membrane currents were monitored by a patch clamp amplifier (EPC-7 or Warner PC501-A). The data were digitized and stored on a videotape recorder for later playback onto a storage oscilloscope (Tektronix 5113) or a chart recorder (Gould RS3200). The data were analyzed with the SCAN program, kindly provided by Dr. J. Dempster, Strathclyde University, UK. The threshold for detection of current events was typically set at the level of 20–25 pA. For the quantitative analysis of the shape of the current events recorded from myocytes the following parameters of the individual membrane currents were calculated: 1) the peak amplitude; 2) the rise time, defined as a time interval between 10% and 90% of the peak amplitude on the upstroke of the current; and 3) the half-decay time, defined as a time interval between the peak and 50% of the peak amplitude on the decaying phase of the current. It should be noted that since the amplitude distribution of the current events is skewed toward smaller sizes (see Fig. 2 *A*), some of the current events are likely to escape detection. Therefore it would be more accurate to use the terms "apparent frequency" and "apparent amplitude" for the quantitative characterization of the current events. However, we will follow previously adopted terminology that is used in similar situations (Kriebel and Gross, 1974; Evers et al., 1989; Vautrin and Kriebel, 1991; Girod et al., 1995).

Microinjection

Conventional microelectrodes were beveled to the tip opening of ~1 μm and backfilled with intracellular solution containing 2000 U/ml acetylcholinesterase (AChE, Sigma) or ACh. A pulse of positive pressure was

applied with Picospritzer (General Valve Co.). We have not attempted to take into account the variations in the volume between different cells. The amount of solution injected into the fibroblast in a typical experiment was estimated to be ~5% of the cell volume (Graessmann et al., 1980).

Treatments to increase $[Ca^{2+}]_i$

We used three different treatments to increase cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$): 1) ATP was dissolved in saline solution and added to the culture medium at the final concentration of 0.3 mM; 2) calcium ionophore ionomycin (Calbiochem) was dissolved in DMSO (10 mM) and added to the culture medium at the final concentration of 10 μM; 3) a conventional microelectrode was used for fibroblast micropuncture. To avoid gross morphological changes of the fibroblast in the vicinity of the contact with myocyte, the site of micropuncture was chosen at a considerable distance (~20–50 μm) from the site of contact between myocyte and fibroblast.

$[Ca^{2+}]_i$ measurements

$[Ca^{2+}]_i$ was measured by the fura-2 ratio imaging technique (Grynkiewicz et al., 1985). Briefly, fibroblasts were incubated in a culture medium containing 6 μM fura-2-AM (Molecular Probes, Portland, OR) for 30 min at room temperature. Coverslips were mounted on the stage of an inverted microscope (Diaphot 300, Nikon) equipped with a cooled CCD camera (model CH200, Photometrics LTD, Tucson, AZ). A Nikon 40X/1.3 NA Fluor DL objective lens was used throughout the experiments. Excitation wavelengths were 340/380 nm with exposure time of 100 ms at each wavelength. Paired digital images at 340- and 380-nm excitation were collected with background subtracted. The size of the measurement box was 20 × 20 pixels, placed at the perinuclear region of each fibroblast. $[Ca^{2+}]_i$ was determined from the ratio (F_{340}/F_{380}) calculated from the pairs using the ratio method (Zheng et al., 1994). Calibration of R_{min} (the limiting value that the ratio can have at zero $[Ca^{2+}]_i$) and R_{max} (the limiting value that the ratio can have at saturating $[Ca^{2+}]_i$) was carried out using standard Ca^{2+} buffers with zero and 39.8 μM free Ca^{2+} (Molecular Probes).

RESULTS

Quantal release of exogenous acetylcholine by fibroblasts

We have previously described an assay for the detection of quantal ACh secretion from CHO cells after cell incubation in the ACh-containing medium (Morimoto et al., 1995). It has been suggested that the quantal ACh release detected by the whole cell *Xenopus* myocytes is due to the endocytic uptake of ACh and the exocytosis of endosome-derived vesicles. However, this hypothesis remained largely untested. For a systematic investigation of the mechanism of ACh uptake and release by nonneuronal cells after incubation in the ACh-containing medium we chose frog fibroblasts (line FT from ATCC). These cells can be maintained under the same culture conditions (L15-based medium, room temperature) that we used for *Xenopus* myocytes, which allows reliable long-term monitoring of secretory activity from ACh-loaded cells. In addition, the FT line of frog fibroblasts has been previously used for the studies of quantal ACh secretion after ACh microinjection into the cytoplasm (Girod et al., 1995). Therefore, the parameters of quantal ACh secretion events following two different protocols of ACh loading can be directly compared.

Exogenous ACh molecules were loaded into frog fibroblasts by incubating the cells in culture medium containing 40 mM ACh for a period of 2 to 20 min. After washing the cells with a fresh medium, a cultured *Xenopus* myocyte was manipulated into contact with the fibroblast (Fig. 1, *A* and *B*). Whole-cell voltage clamp recordings from the myocyte started 5 min after the end of incubation with ACh. These recordings revealed fast inward currents (Fig. 1, *C* and *D*) that resembled miniature endplate currents (mepcs) found at developing neuromuscular synapses (Xie and Poo, 1986). No current events were detected in recordings from myocytes in contact with control fibroblasts that were not incubated with ACh ($n = 5$). Moreover, the mepc-like events were completely abolished by bath-application of d-tubocurarine, a drug known to block nicotinic ACh receptors in the myocyte ($n = 4$). Thus, these current events were due to the release of packets of ACh from the fibroblasts. These observations are in agreement with previously reported quantal ACh secretion from CHO cells loaded with exogenous ACh using a similar incubation protocol (Morimoto et al., 1995).

Secretion of exogenous ACh is mediated by the endocytic recycling pathway

The amplitude distribution of the current events was skewed toward smaller sizes (Fig. 2 *A*), similar to that found at developing neuromuscular synapses (Evers et al., 1989), central synapses (Bekkers et al., 1990), and nonneuronal cells injected with exogenous ACh (Girod et al., 1995). The variation in amplitude of current events appears to result from different amounts of ACh in each secreted packet, rather than from a local variation in the density of ACh receptors on the myocyte surface (Evers et al., 1989; Girod et al., 1995). The recordings from fibroblasts started at later times after the end of incubation showed a progressive decline in the initial frequency of events (Fig. 2 *B*), suggesting that most of the ACh had already been secreted. Therefore, it appears that secretion of ACh occurs spontaneously in the absence of myocyte contact. The initial frequency and the mean amplitude of the current events increased with the duration of ACh incubation (Fig. 1 *C*). No events with amplitudes higher than 200 pA were recorded from myocytes incubated with ACh for 2 min ($n = 6$). Five minutes after the end of a 10-min incubation, the mean amplitude and mean frequency of the current events were 240 ± 16 pA and 6.5 ± 1.0 events/min (mean ± SE, $n = 14$), respectively. For a given duration of incubation, both the frequency and the mean amplitude showed a gradual decline with time (Fig. 2, *C* and *D*).

To investigate whether ACh penetration into fibroblasts during incubation and subsequent secretion were specific to ACh, we compared the time course of ACh secretion with release of ^{14}C-sucrose, a membrane-impermeant marker of bulk fluid-phase endocytosis. After a 10-min incubation in a solution containing ^{14}C-sucrose, the amount of radioac-

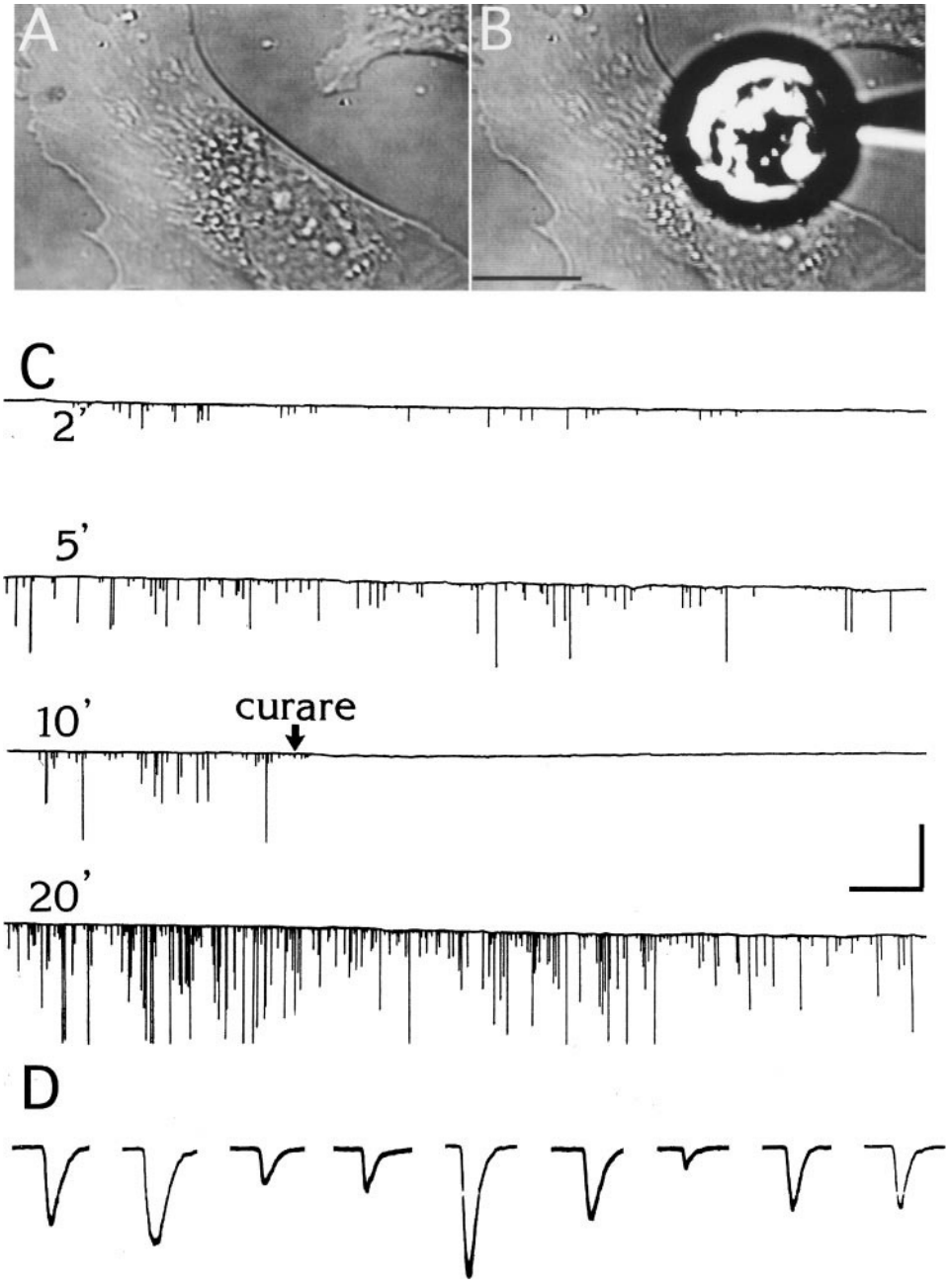


FIGURE 1 Secretion of exogenous acetylcholine from fibroblasts. Microscopic images of a cultured fibroblast before (*A*) and after (*B*) a *Xenopus* myocyte was manipulated into contact. Bar = 20 μm. (*C*) Examples of membrane currents recorded from myocytes in contact with fibroblasts that were incubated in culture medium containing 40 mM ACh for a period of 2, 5, 10, or 20 min, respectively. Quantal release of ACh, as shown by pulsatile inward currents (downward deflections), was monitored by whole-cell voltage-clamp recording from the myocyte at resting membrane potential (−70 mV, filtered at 150 Hz). All recordings started 5 min after the end of incubation. Bath application of d-tubocurarine (1 mM, Sigma, *arrow*) completely abolished the current events. (*D*) Samples of current events during the period 6–8 min after the onset of recording shown at a higher time resolution (filtered at 2 kHz). Scales: 400 pA, 100 s in (*C*) and 200 pA, 40 ms in (*D*)

tivity released from the fibroblasts decreased with time (Fig. 2 *F*). The half-decay time of sucrose secretion was 12.7 ± 0.2 min (mean ± SE, $n = 6$). For fibroblasts incubated with ACh for 10 min, the amount of secreted ACh, expressed as the product of the frequency and mean amplitude of the current events (Fig. 2 *E*), declined with a mean half-decay time of 12.2 ± 1.1 min (mean ± SE, $n = 14$), a value close to the half-decay time of sucrose secretion. A similar decline in the rate of secretion (half-decay time 10.0 ± 1.5 min, $n = 4$) was observed after incubation of fibroblasts with carbachol, a nonhydrolyzable ACh analog, indicating that ACh hydrolysis did not significantly contribute to the decline of the amount of released ACh. Moreover, when the incubations with sucrose ($n = 3$) or ACh ($n = 5$) were carried out at 4°C, a condition known to inhibit endocytosis, an insignificant amount of ^{14}C-sucrose release and no detectable ACh release were observed after incubation. Thus ACh uptake and secretion by fibroblasts appear to occur nonspecifically by the well-documented fluid-phase endocytic pathway. The observed gradual decline in the frequency and amplitude of the current events with time during the recording (Fig. 2, *C* and *D*) is consistent with idea that at least some of the ACh-containing endocytic vesicles did not undergo exocytosis directly after formation, but rather fused with intermediate (endosomal) compartments. Progressive reduction of the ACh concentration within endosomal compartments after the end of incubation would result in a gradual decrease in the amount of ACh in the

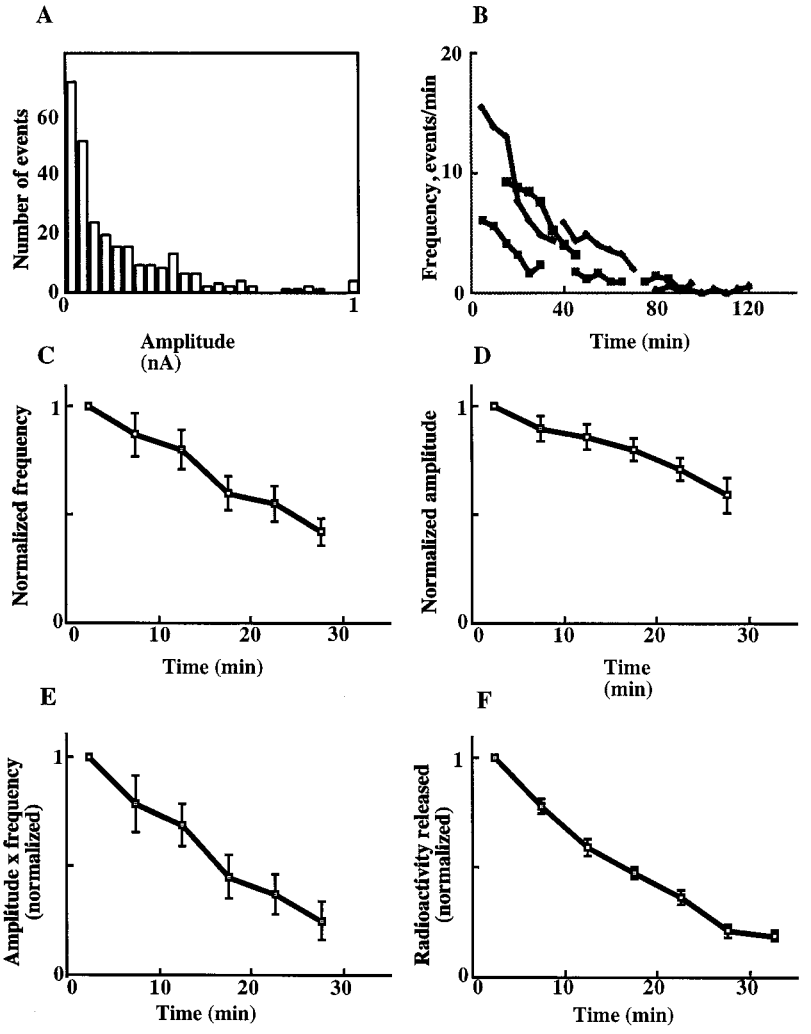


FIGURE 2 Parameters of mepc-like current events. (*A*) The distribution of the amplitudes of mepc-like events recorded from a myocyte in contact with an ACh-incubated fibroblast. Data from a typical recording during a period 5–15 min after the end of ACh incubation. (*B*) Changes in the frequency of mepc-like events with time observed in different myocytes in contact with ACh-incubated fibroblasts. Spontaneous ACh secretion was recorded from different fibroblasts that had been incubated in culture medium containing 40 mM ACh for 10 min. The onset time of each recording after the end of the incubation period (time 0) varied among different recordings. Data from the same recordings are connected by the line. Frequency was calculated as the mean value over 5-min intervals. (*C*) and (*D*) Changes in the frequency and amplitude of the current events with time after the onset of recording, normalized to the values at the beginning of the recording (5 min after the end of a 10-min incubation in 40 mM of ACh). Data from 14 fibroblasts were normalized for each cell before averaging. (*E*) Decline in the spontaneous ACh secretion from fibroblast, which is represented by the product of the mean frequency and amplitude for each 5-min interval. The data are from the same set of experiments as those shown in (*C*) and (*D*). Calculations were performed for each cell before averaging. (*F*) Release of ^{14}C-sucrose after endocytic uptake by the fibroblasts. Parallel fibroblast cultures were incubated for 10 min in solution containing 50 μCi/ml of ^{14}C-sucrose. The radioactivity released into the culture medium was measured at 5-min intervals and normalized for each experiment to the secretion during the first 5 min. Data from six experiments. Bars indicate mean ± SE

endosome-derived recycling vesicles and a smaller amplitude of the current events. The frequency of the detected fusion events would also decrease, as more events will have amplitudes less than the detection threshold. Assuming that the secretion of ACh is uniform on the fibroblast surface, and that the area of myocyte-fibroblast contact is on the order of 10 μm^2, or 0.3% of the total surface area of a typical fibroblast, the average frequency of spontaneous secretion from the entire fibroblast would be ~2000/min. This rate of spontaneous exocytosis is consistent with the estimated rate of membrane recycling under normal conditions (Griffiths et al., 1989; Major et al., 1993).

Penetration of positively charged ACh molecules through the plasma membrane of fibroblasts in the absence of specific transport activity is expected to be inefficient. Somewhat surprisingly, we detected ACh accumulation in the cytoplasm of fibroblasts incubated with ACh. Specifically, when fibroblasts were gently poked with a sharp microelectrode, a long-lasting current 20–300 pA in amplitude, was recorded from the myocyte brought into contact with fibroblast (Fig. 3 *A*). This current was eliminated (Fig. 3 *B*) when fibroblasts were injected with acetylcholinesterase (AChE, final cytoplasmic concentration 100 U/ml) ($n = 5$), before incubation with ACh. This suggests that the observed current was due to the efflux of cytoplasmic ACh from fibroblasts into the culture medium through the site of microelectrode penetration. Despite the lack of detectable ACh in the cytoplasm of these cells, quantal ACh secretion was still observed (Fig. 3 *B*). To further address a possible role of cytoplasmic ACh in the secretion of ACh packets, fibroblasts were injected with ACh through conventional microelectrodes. Cytoplasmic ACh concentration in these experiments was estimated to be ~1 mM. Myocytes were manipulated into contact with fibroblasts 10 min after injection. No current events were observed in whole-cell patch clamp recordings from myocytes ($n = 7$). Micropuncture of these "silent" fibroblasts with a sharp micropipette resulted in a long-lasting inward current in myocytes with amplitude on the order of 200-1000 pA (Fig. 3 *C*), indicating that the cytoplasmic concentration of ACh in these cells was ~1 order of magnitude higher than that in the cytoplasm of fibroblasts incubated with ACh. Secretion events, however, were observed exclusively from fibroblasts incubated with ACh. Therefore, although during incubation

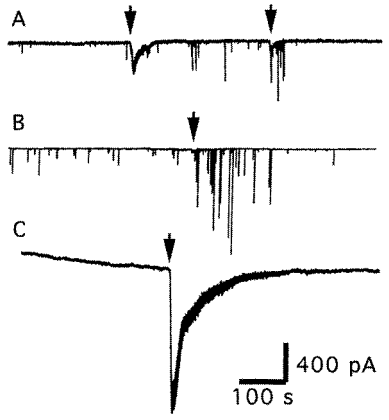


FIGURE 3 The role of cytoplasmic ACh in quantal ACh secretion. (*A*) Example of membrane current recorded from myocyte in contact with fibroblast that was incubated in culture medium containing 40 mM ACh for 10 min. Recording started 5 min after the end of incubation. Quantal release of ACh (downward deflections) was observed. The fibroblast was poked with a sharp microelectrode (*arrows*), which resulted in the inward current in the myocyte with slow rise and decay times, suggesting leakage of the cytoplasmic ACh into the culture medium. This slow current was eliminated when, before incubation with ACh, fibroblasts were injected with acetylcholine esterase (final cytoplasmic concentration ~100 U/ml) (*B*) Note that although no cytoplasmic ACh is detected in this experiment, quantal ACh secretion can still be observed in these cells. (*C*) Example of recording from myocyte brought into contact with ACh-injected fibroblast. ACh concentration in the micropipette used for injection was 20 mM. The concentration of ACh in the cytoplasm of injected fibroblasts was estimated to be ~1 mM. The recordings were started 10 min after injection. No quantal ACh release was observed. Micropuncture of the fibroblast with a sharp microelectrode resulted in a large (~1 nA) inward current with slow time course. The amplitude of this current was typically an order of magnitude higher than that induced by the micropuncture of fibroblasts, incubated with ACh (*A*).

exogenous ACh penetrates through the plasma membrane of fibroblasts and is present in the cytoplasm at detectable levels, cytoplasmic ACh does not contribute to the quantal ACh secretion from fibroblasts.

It should be noted that in our previous study (Girod et al., 1995), as well as in the present work (see below), we were able to detect quantal ACh secretion from fibroblasts after ACh microinjection. In these experiments the concentration of ACh in the cytoplasm was ~25 mM, that is, ~25-fold higher than in the experiment, illustrated on Fig. 3 *C*. This suggests that the amount of ACh reaching the releasable vesicular pool depends on the cytoplasmic ACh concentration. At the 1-mM ACh concentration, this amount is too small to induce detectable mepcs in myocytes.

Taken together, the above results strongly support the notion that mepc-like events recorded after incubation of fibroblasts in ACh-containing medium reflect a spontaneous exocytosis of vesicles involved in endosomal membrane recycling.

Dynamics of individual exocytotic events in fibroblasts

To study the dynamics of individual exocytotic events in fibroblasts we analyzed the shape of the currents recorded from myocytes, the approach used in the studies of secretion dynamics (Girod et al., 1993). The majority of the current events were similar in shape to the mepcs recorded from myocytes in the developing *Xenopus* neuromuscular synapse. The rise and half-decay times of the current events recorded from fibroblasts were 4.2 ± 0.6 ms and 6.5 ± 0.7 ms (mean ± SE) respectively. A significant fraction of current events (~10%) in recordings from fibroblasts was atypical in shape (Fig. 4). These current events had unusually large rise and/or decay time, and were irregular in shape. Some of the events were characterized by the small "foot" preceding an abrupt downstroke. Similar atypical current events have been detected in the recordings from neuronal cells (Kriebel and Pappas, 1987; Chow et al., 1992) and ACh-injected fibroblasts (Girod et al., 1995). The average rise and half-decay time of the current events, as well as the frequency of atypical events, are identical to those previously reported for fibroblasts injected with ACh (Table 1). It has been previously demonstrated that these atypical events are most likely due to unusually slow transmitter release during exocytotic fusion rather than inhomogeneous spatial properties of the sensor used for ACh detection or the temporal overlap of individual exocytotic events (Girod et al., 1995).

Sensitivity of the endosomal membrane recycling to the elevation of $[Ca^{2+}]_i$

The assay for detection of exocytotic events in nonneuronal cells was used to examine the effect of cytoplasmic Ca^{2+}

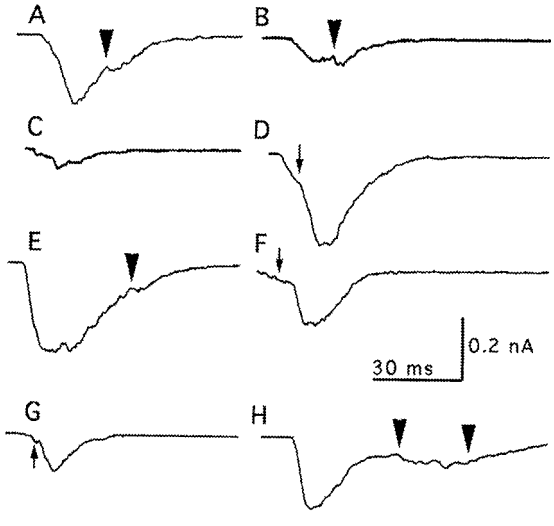


FIGURE 4 Examples of atypical current events recorded from myocytes brought into contact with ACh-secreting fibroblasts. Incubation of fibroblasts with ACh and recording of membrane currents were performed as in Fig. 1. Some of the current events were characterized by an unusually slow rate of rise, or by long decay time, and typically were irregular in shape. Some of the events were characterized by a slow "foot" preceding the abrupt upstroke (*arrows* in *D, E,* and *G*). Note multiple notches on the current traces in *A, B, E,* and *H* and unusually long decay time in *H* (*arrowheads*).

TABLE 1 Dynamics of current events in recordings from fibroblasts loaded with exogenous ACh either by incubation in the ACh-containing medium or by injection

	Rise Time (ms)	Half-Decay Time (ms)
Incubation with ACh	4.2 ± 0.6	6.5 ± 0.7
ACh injection*	3.8 ± 0.7*	6.2 ± 0.9*

Fibroblasts were loaded with ACh by a 10-min incubation in the ACh-containing medium. Mepc-like current events were recorded for a period of 10 min. Recordings started 5 min after the end of incubation. Data are presented as mean ± SE of 14 different experiments.
*Data are from Girod et al., 1995.

($[Ca^{2+}]_i$) elevation on endosomal membrane recycling. Treatment of fibroblasts with ATP, which is known to induce an increase in $[Ca^{2+}]_i$ due to opening of ATP-dependent Ca^{2+} channels and Ca^{2+} release from intracellular stores (Salter and Hicks, 1994), resulted in an increase in the frequency of spontaneous ACh secretion events (Fig. 5 *A*). The elevation of $[Ca^{2+}]_i$ was monitored directly by using a $[Ca^{2+}]_i$-sensitive dye, fura-2. After ATP application, $[Ca^{2+}]_i$ increased from a resting level of 108 ± 7 nM (mean ± SE, $n = 9$) to 417 ± 49 nM (mean ± SE, $n = 39$) at 2–5 min after the start of ATP incubation. Preincubation of fibroblasts with BAPTA-AM (20 μM), a membrane-permeable Ca^{2+} buffer, completely abolished the effect of ATP on the frequency of secretion events, indicating that the effect of ATP was due to the increase in $[Ca^{2+}]_i$. Similarly, treatment of fibroblasts with ionomycin, a Ca^{2+} ionophore known to trigger Ca^{2+} influx through the plasma membrane as well as Ca^{2+} release from internal stores, also resulted in an increase in the frequency of spontaneous ACh secretion (Fig. 5 *B*). Furthermore, micropuncture of the fibroblasts with a micropipette induced transient bursts of quantal ACh secretion (Fig. 5 *C*). The bursting secretion depended on Ca^{2+} influx through the plasma membrane, since micropuncture had no effect on the frequency of the ACh secretion when the external medium was supplemented with 10 mM EGTA. All three methods of raising $[Ca^{2+}]_i$ resulted in an increase in the mean amplitude of current events recorded from myocytes. The mean amplitude of current events 5 min after application of ATP was 1.16 ± 0.18 (SE, $n = 11$) times that observed before application, 10 min after application of ionomycin it was 1.18 ± 0.26 (SE, $n = 6$) times that before application, and 2 min after micropuncture it was 1.51 ± 0.24 (SE, $n = 9$) times that before micropuncture. The slight increase in the mepc amplitude upon elevation of $[Ca^{2+}]_i$ may reflect Ca^{2+}-dependent vesicle-vesicle fusion before exocytosis (Terasaki et al., 1997). The observed amplitude increase was too small to explain the marked increase in the frequency of current events upon elevation of $[Ca^{2+}]_i$.

A similar Ca^{2+}-dependence of quantal neurotransmitter release has been previously reported for frog fibroblasts injected with exogenous ACh (Girod et al., 1995). These results are consistent with an idea that both protocols (incubation with ACh and ACh injection in the cytoplasm) result in the loading of ACh into similar membrane compartments.

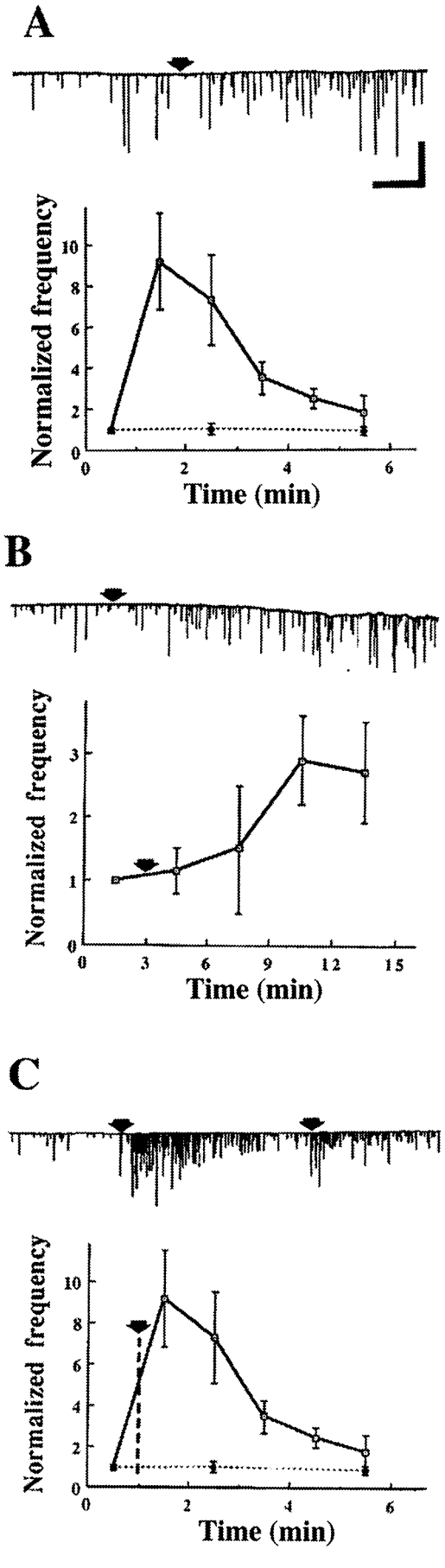
Penetration of cytoplasmic ACh into endosomal membrane compartments

To further address the nature of the membrane compartments responsible for accumulation of cytoplasmic ACh and quantal ACh secretion, we pressure-injected ACh into the fibroblast via conventional microelectrodes. The concentration of ACh in the cytoplasm of injected cells was estimated to be ~25 mM, which is ~25-fold higher than that in experiment, illustrated in Fig. 3 *C*. As before, a *Xenopus* myocyte was manipulated into contact with the injected fibroblast to detect secretion of ACh. In agreement with a previous report (Girod et al., 1995), spontaneous quantal ACh secretion from injected fibroblasts could be detected immediately after the start of recording. The frequency of secretion events gradually increased with time after injection and reached a plateau in ~30 min (data not shown) (Girod et al., 1995).

Topological connectivity between the vesicular compartments that accumulated cytoplasmic ACh after microinjection and those derived from endocytic pathways was assessed by incubating ACh-injected fibroblasts in the culture medium containing AChE (100 U/ml), which is expected to enter the endocytic pathway as a bulk fluid phase marker. A significant reduction in the frequency and the amplitude of the current events was found after AChE-incubation (Fig. 6, *A* and *C*), suggesting that intracellular injected ACh molecules accumulated into compartments that are rapidly accessible to AChE, which entered the cell through the endocytic pathway. The observed reduction in the frequency and amplitude of current events in these experiments was not due to the extracellular action of AChE on the released ACh, since AChE at the concentration used in these experiments (100 U/ml) will not significantly hydrolyze extracellular ACh at the millisecond time scale to affect the amplitude of the current events. The lack of extracellular effect of AChE was confirmed by the finding that similar treatment with AChE did not affect the frequency or the amplitude of mepcs at developing *Xenopus* neuromuscular synapses (Fig. 6, *B* and *D*), where presynaptic nerve terminals undergo little endocytic activity at the resting state.

DISCUSSION

In neuronal cells, ACh is synthesized in the cytoplasm at the nerve terminal (Parsons et al., 1983), transported into synaptic vesicles by a specific transporter (Alfonso et al., 1993), and secreted during the transient elevation of cytoplasmic Ca^{2+} induced by an action potential [a possible exception to this generally accepted model of neurotransmitter release may include an electrical organ of *Torpedo*, where a Ca^{2+}-dependent protein called mediatophore may be involved in ACh translocation through the plasma membrane (Falk-Vairant et al., 1996a)]. In addition to the Ca^{2+}-dependent secretion induced by the action potential, neurotransmitters are also secreted spontaneously in the quantal fashion (Chow and Poo, 1985; Evers et al., 1989), even in



the absence of the contact with the postsynaptic target (Kraszewski et al., 1995; Dai and Peng, 1996). The spontaneous quantal neurotransmitter secretion is likely to be important in the development of the initial contact between the presynaptic neuron and the postsynaptic target into a mature synapse (Kidokoro, 1984; Xie et al., 1997).

The physiological recordings from postsynaptic cells indicate that in many synapses responses to individual quanta display large variations in the mepc amplitude (Muniak et al., 1982; Parsons et al., 1983; Vautrin and Kriebel, 1991). The origin of this variability, although central to our understanding of the mechanisms of synaptic transmission, remains controversial. The variations in the mepc amplitudes in some cases may be explained by the variations in the number of postsynaptic receptors (Nusser et al., 1997). However, in cholinergic synapses presynaptic mechanisms are also clearly involved. In some cholinergic synapses there is significant morphological, biochemical and physiological evidence for the populations of vesicles that differ in the amount of ACh (Williams, 1997). In addition, a number of manipulations, such as electrical activity (Searl et al., 1990), β-adrenergic stimulation (Parsons et al., 1983), activation of cAMP-dependent pathway (Falk-Vairant et al., 1996), overexpression of vesicular ACh transporter (Song et al., 1997), treatment with tetanus or botulinum toxins (Vautrin, 1992; Herreros et al., 1995), hypertonic solution (Kriebel et al., 1996), or drugs that interfere with ACh metabolism (Williams, 1997), can modify the quantal size at the cholinergic synapses.

Spontaneous secretion of ACh has also been detected in nonneuronal cells (Del Castillo and Katz, 1954; Kriebel, 1980; Dan and Poo, 1992; Girod et al., 1995; Falk-Vairant et al., 1996b). The membrane compartments responsible for ACh secretion in nonneuronal cells may represent a primitive vesicular system for quantal secretion of neurotransmitters. In the present study we investigated the nature of

FIGURE 5 Effect of $[Ca^{2+}]_i$ elevation on quantal ACh release from fibroblasts. Current traces are examples of recordings from myocytes in contact with fibroblasts, similar to those shown in Fig. 1. (*A*) ATP was added to the external medium 5 min after the start of recording (*arrow*) at a final concentration of 0.3 mM. The data were normalized to the frequency of secretion events before ATP addition for each fibroblast before averaging. A transient increase in secretion frequency was observed after ATP application (*solid lines*, data from 11 experiments). Preincubation of fibroblasts with acetoxymethyl ester of BAPTA (1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid) (BAPTA-AM, 20 μM) for 20 min completely abolished the ATP-induced increase in the frequency of secretion events (*dashed line*, data from seven experiments). (*B*) Effect of the Ca^{2+} ionophore ionomycin on the frequency of secretion events. Ionomycin was added to the external medium at the time marked by the arrow at a final concentration of 10 μM. The data were normalized to the frequency before ionomycin addition for each fibroblast before averaging. Data from six experiments. (*C*) Effect of micropuncture on the frequency of secretion events. The time of micropuncture is marked by the arrow. A transient increase in frequency was observed for micropuncture in normal medium (*solid lines*, $n = 9$). No increase was observed in EGTA-supplemented (10 mM) medium (*dashed line*, $n = 4$). Scale, 400 pA and 100 s.

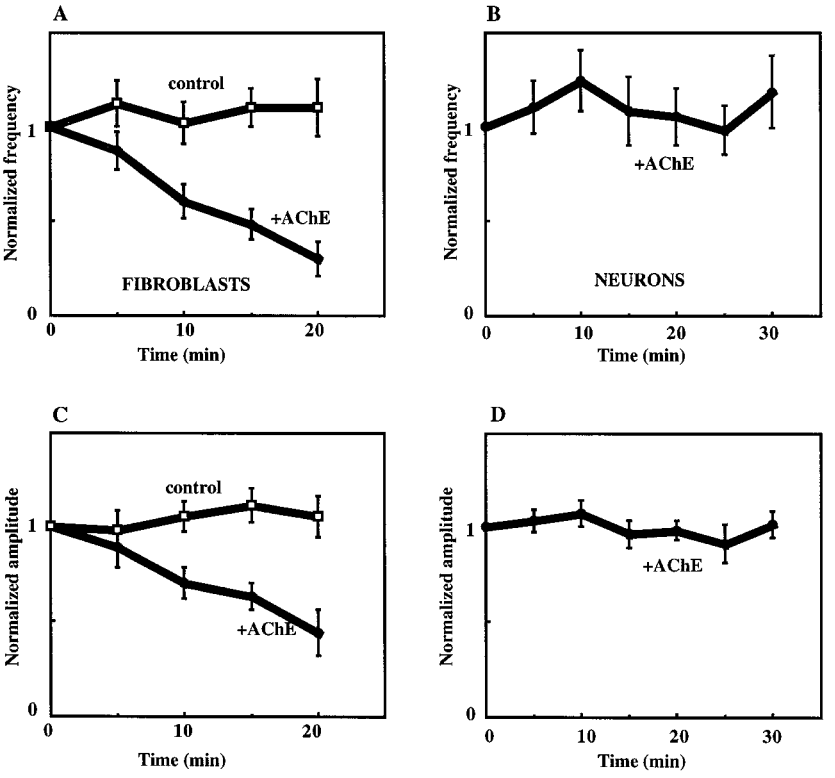


FIGURE 6 (*A*) and (*C*) Fibroblasts were injected with ACh through conventional microelectrodes. The concentration of ACh in the cytoplasm of injected cells was ~25 mM. Recordings from injected fibroblasts using whole-cell myocytes started 30 min after injection and continued for a period of 25 min. In each experiment the average frequency and amplitude of the current events during 5-min intervals were normalized to those at the beginning of the recording. Data are presented as mean ± SE. Both frequency and amplitude of the current events remained at a relatively stable level throughout a period of recording (*open squares*, data from nine experiments). Addition of AChE (100 U/ml) to the culture medium 30 min after ACh injection resulted in the rapid decline in the average frequency and amplitude of current events (*filled circles*, data from eight experiments). Identical treatment with AChE did not affect the average frequency (*B*) or the average amplitude of the mepcs (*D*) recorded at the spontaneously formed *Xenopus* neuromuscular synapses (data from seven experiments).

these membrane compartments in fibroblasts. Two methods of ACh loading into fibroblasts were used. In the first approach, fibroblasts were incubated in the culture medium containing ACh, and individual secretion events were revealed by recordings from whole-cell *Xenopus* myocytes (Morimoto et al., 1995; Falk-Vairant et al., 1996b). Although it has been assumed that the vesicles responsible for spontaneous neurotransmitter release in these experiments were endosome-derived, this hypothesis was largely untested. By using a number of criteria we demonstrate that spontaneous ACh secretion events recorded from fibroblasts are indeed due to the exocytosis of the vesicles participating in the endosomal membrane recycling: 1) although exogenous ACh penetrated through the fibroblast plasma membrane during incubation and was present in the cytoplasm at detectable levels, cytoplasmic ACh did not contribute to the quantal ACh secretion; 2) the time course of ACh secretion was similar to that of ^{14}C sucrose and the nonhydrolyzable ACh analog carbachol; 3) no ACh secretion was observed when endocytic uptake of ACh was blocked by incubating cells with ACh at 4°C; and 4) progressive decline in the frequency and average amplitude of current events with time during recording is consistent with the existence of the intermediate (endosomal) compartment. Taken together, these data suggest that exogenous ACh is nonspecifically taken up by the cells by well-documented fluid phase endocytosis. Although our results do not completely exclude the possibility that during a brief (10 min) period of incubation ACh reaches the late endosomal/lysosomal compartments, they are more consistent with the early endosomal origin of the exocytotic vesicles spontaneously releasing ACh.

Endosomal membrane trafficking in nonneuronal cells is poorly understood in comparison with synaptic vesicle recycling. The vesicles participating in the retrieval pathway from endosomes to the plasma membrane have not been identified biochemically. Moreover, the exocytosis of these vesicles is currently beyond the resolution of capacitance (Neher and Marty, 1982), or amperometric measurements (Wightman et al., 1991). Our results support the previous finding of the Ca^{2+}-dependence of the endosomal recycling pathway in nonneuronal cells (Morimoto et al., 1995). These data are in general agreement with a large body of experimental evidence indicating that some steps of the vesicular trafficking in nonneuronal cells are Ca^{2+}-dependent (Steinhardt et al., 1994; Coorsen et al., 1996; Ninomiya et al., 1996).

To detect the secretory events in fibroblasts following endocytic ACh uptake, we adopted the approach used previously for characterization of the quantal ACh secretion after ACh injection into the cytoplasm of nonneuronal cells. We systematically analyzed the frequency, amplitude, rise time, half-decay time, the shape of the currents events, and sensitivity of ACh secretion to elevation of Ca^{2+}. These experiments revealed a striking similarity between quantal ACh secretion following endocytotic uptake and that described previously for fibroblasts injected with ACh. The simplest interpretation of these data is that the vesicles, responsible for uptake of exogenous cytoplasmic ACh, are associated with the constitutive endocytic recycling pathway. This idea was further supported by the rapid accessibility of the ACh-accumulating compartments in fibroblasts injected with ACh to extracellularly added AChE.

The mechanism of cytoplasmic ACh uptake by endocytic vesicles in nonneuronal cells remains to be investigated. There is no direct experimental evidence for the expression of the ACh transporter in fibroblasts. We observed a slow penetration of ACh through the plasma membrane following incubation of fibroblasts in ACh-containing medium (Fig. 3 *A*). Similarly, the rate of cytoplasmic ACh packaging into endocytic vesicles was found to be a few orders of magnitude slower than that for synaptic vesicles. These data suggest that cytoplasmic ACh loading into endocytic vesicles is not mediated by specific transport activity. The packaging of cytoplasmic ACh into vesicles may be facilitated by the acidic pH in the endosomal compartments (Dan et al., 1994). Our data also allow for a rough estimate of the ACh concentration in the membrane compartments following ACh injection into the cytoplasm. The concentration of ACh in the endocytic compartments after incubation in the ACh-containing medium does not exceed that in the culture medium (40 mM). Since two protocols of ACh loading into membrane compartments (injection and incubation) result in the mepcs of similar amplitude (Girod et al., 1995), the ACh concentration in the membrane compartments following ACh injection also does not exceed 40 mM. This estimate again suggests that the mechanisms of ACh penetration into vesicular compartments in nonneuronal cells and into neuronal synaptic vesicles are different. Further in vitro studies are needed to determine the effects of the lipid composition of vesicles and pH gradient on the packaging of ACh into these membrane compartments.

In summary, we show that the endocytic compartments in nonneuronal cells are able to accumulate and secrete cytoplasmic ACh in a Ca^{2+}-dependent fashion, thus imitating the basic functions of synaptic vesicles. Our results suggest that the ubiquitous endosomal membrane recycling pathway may contribute to spontaneous quantal neurotransmitter secretion in neurons. Indeed, ACh is present in the cytoplasm of neuronal cells (Parsons et al., 1983), and endocytic recycling pathway operates in any type of cell, including neurons (Kraszewski et al., 1995; Dai and Peng, 1996). Extrapolation of our results to neuronal cells indicates that ACh molecules are likely to penetrate into endocytic membrane compartments. Constitutive exocytosis of the endosome-derived vesicles is expected to result in a detectable change of the membrane potential in the postsynaptic cell. Thus, the small amplitude mepcs observed at the neuromuscular junction, which presumably reflect the exocytosis of vesicles with unusually low ACh content (Parsons et al., 1983), may reflect the exocytosis of constitutively recycling vesicles. The absence of defined quanta at the developing *Xenopus* neuromuscular synapse (Kidokoro, 1984; Evers et al., 1989), re-innervated mouse neuromuscular junction (Muniak et al., 1982) and central synapses (Bekkers et al., 1990) may reflect the high proportion of "immature" synaptic vesicles, which are similar in molecular composition to constitutive recycling vesicles in that they lack some molecular components specific to synaptic vesicles.

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