Enhancement of Asynchronous and Train-Evoked Exocytosis in Bovine Adrenal Chromaffin Cells Infected With a Replication Deficient Adenovirus

Ramachandran Thiagarajan, Jennifer Wilhelm, Teclemichael Tewolde, Yingjie Li, Mark M. Rich, and Kathrin L. Engisch

Department of Physiology, Program in Neuroscience, Department of Neurology, Center for Neurodegenerative Diseases, Emory University School of Medicine, Atlanta, Georgia

Submitted 31 March 2005; accepted in final form 13 July 2005

INTRODUCTION

Bovine adrenal chromaffin cells are often used as a model system to study ion channel function and neurotransmitter release (Artalejo et al. 1991; Bader et al. 2002; Burgoyne and Morgan 2003; Fenwick et al. 1982; Kobayashi et al. 2002; Martin 2003). Chromaffin cells have Na+-dependent action potentials, express voltage-gated K+ channels and Ca2+ channels, and express many of the proteins implicated in neurotransmitter release, including NSF-SNARE proteins, munc13, synaptotagmin, and Rab3A. A common approach to study the function of an ion channel or synaptic protein is to express wild-type protein. Unfortunately, nondividing chromaffin cells are difficult to transfect with high efficiency. Specialized protocols for calcium phosphate (Holz et al. 1994; Wick et al. 1993), electroporation (Weiss et al. 2000), and the Gene Gun (Li et al. 2003) have been modestly successful, but easier and better means to induce expression of exogenous genes in chromaffin cells would be of great benefit to the field.

A number of recent studies have employed Semliki Forest Virus to express synaptic proteins in chromaffin cells (reviewed in Retig and Neher 2002; for 2 recent examples, see Nagy et al. 2004; Yizhar et al. 2004). The first descriptions of chromaffin cells infected with SVF reported that up to 90% of cells could be infected with no toxicity and only minor alterations in electrophysiological properties (Ashery et al. 1999; Duncan et al. 1999). However, Pan et al. (2002) subsequently showed that calcium currents were reduced by almost half, and exocytosis declined with repeated stimulation in perforated-patch recordings.

Another possible gene delivery vector is recombinant, replication deficient adenovirus. Adenovirus is being aggressively studied for its potential use in gene therapy because it can infect nondividing cells such as neurons (reviewed in Romano et al. 2000). It was shown many years ago that cells of the adrenal gland, including cortical and medullary cells, robustly take up adenovirus during a systemic infection (Hoenig et al. 1974; Margolis et al. 1974). More recently, Li et al. (2002) showed that chromaffin cells can be infected by adenovirus in vitro. Adenovirus has great potential for inducing gene expression in chromaffin cells because it infects a large proportion of cells with little cell death or morbidity even at 48 h post infection. A few studies have performed electrophysiological recordings in neurons infected with adenovirus and found no obvious changes in voltage-gated K+ currents (Slack et al. 1996), resting potential, cell input resistance, or action potential properties (Smith et al. 1997). These reports suggest adenovirus may have minimal effects on chromaffin cell physiology. However, many electrophysiological properties, including Ca2+ currents and neurotransmitter release, have not been examined in adenovirus infected neurons or chromaffin cells.

With the exception of our previous study using adenovirus to increase expression of Rab3A in bovine chromaffin cells (Thiagarajan et al. 2004), we are unaware of any report that has combined adenovirus infection of chromaffin cells with whole cell patch-clamp recording. In our previous study, we briefly
reported that the control adenovirus caused alterations in calcium currents and exocytosis. Here for the first time we describe in detail the electrophysiological changes that occur in bovine chromaffin cells infected with adenovirus and examine possible mechanisms.

METHODS

Generation of GFP-IREs-β-galactosidase adenovirus

The sequence for GFP was inserted into a multiple cloning site in the pAdLink plasmid containing a CMV promoter-multiple cloning site-IREs-β-galactosidase construct (Gonzalez et al. 1999). Recombinant, E1-deleted replication-defective adenovirus was generated by homologous recombination with Cla-I-digested, partial E3-deleted di327 Ad5 backbone (Kraner et al. 1999). Adenovirus was plaque purified and grown as described in Kraner et al. (1999).

Culture, adenovirus infection, and electroporation of bovine chromaffin cells

Chromaffin cells were isolated from adult bovine adrenal glands by collagenase digestion and plated on collagen-coated glass coverslips as previously described (Thiagarajan et al. 2004; Vitale et al. 1991). On day 3 of culture, frozen virus was thawed on ice and diluted in plating medium consisting of Dulbecco’s modified Eagle’s medium with 25 mM HEPES (Invitrogen), 10% fetal bovine serum (Media- tech), and antibiotics and mitotic inhibitors (0.01% streptomycin, 0.01% penicillin, 0.001% gentamycin, 10 μM cytosine arabinoside, and 10 μM fluorodeoxyuridine; Sigma-Aldrich).

For each culture, we empirically determined the viral dilution that induced GFP fluorescence in ~2/3 of the cells, to account for time-dependent degradation of viral titer and variations in viral titer between virus preparations. In a separate experiment, the number of plaque forming units for a typical dilution was determined in 293 cells and was 0.5 × 10^5 plaque forming units/ml. Cells were fed by replacing half of the medium with fresh plating medium (control cells) or with fresh medium containing serial dilutions of virus. Electrophysiological experiments were performed in “bright” GFP cells 48 h after infection. In these experiments, we did not have the means to quantitate the GFP fluorescence but used the criterion that the GFP fluorescence remained visible when the white transmitted light source was on. The medium was not changed after virus addition, but similar effects were observed 48 h after infection when the virus-containing medium was completely replaced with fresh medium at 5 h after infection (data not shown).

Cells were electroporated using a Nucleofector device according to the manufacturer’s protocol (Amaxa). Nucleofection requires that cells be in suspension. We used freshly isolated cells because cells plated for 3 days and then dispersed into suspension fared poorly after nucleofection. Therefore we were unable to exactly match the experimental conditions of viral infection. However, cells infected with virus at the time of plating showed the same effects as cells infected with virus on day 3 (see RESULTS). At the end of the dissociation procedure, 2 × 10^6 cells were resuspended in 100 μl of Nucleofection Solution VPI-1003. pMaxGFP (2 μg) was added before transferring the cell suspension to an electroporation cuvette. Cells were electroporated using program A-33. To keep the time of gene expression the same as that in adenovirus experiments, perforated-patch recordings were performed at 48 h postnucleofection on day 2 of culture. For choosing “bright” GFP-positive cells, a 25% neutral density filter was placed in the path of the UV light source, to equalize the brightness levels of pMaxGFP, which is from the copepod Pontellina plumata, to that of the Aequorea victoria GFP, expressed by adenovirus-infected cells. Approximately 30% of cells expressed pMaxGFP 48 h post-nucleofection (data not shown).

Inactivation of adenovirus

The β-galactosidase-IREs-GFP adenovirus was inactivated by UV irradiation according to the procedure of Cotten et al. (1992), with minor changes. Adenovirus was incubated on ice in 0.165 μg/μl 8-methoxypsoralen (Sigma-Aldrich) at 4 cm from a 365 nm UV light source for 30 min. Inactivation was confirmed by comparing the percentage of GFP or β-gal-positive HEK 293 cells after infection with serial dilutions of UV irradiated virus and viral dilutions of intact virus. The inactivation procedure produced virus that was <1 ten-thousandth as potent as the starting material at inducing expression of GFP or β-gal in HEK 293 cells. GFP fluorescence was never observed in chromaffin cell cultures infected with UV-irradiated adenovirus.

Immunohistochemistry

Chromaffin cells were processed for immunofluorescence 3–4 h after infection with intact adenovirus or UV-irradiated adenovirus. Cells were fixed in 4% paraformaldehyde for 10 min, permeabilized in 0.2% saponin for 2 min, and incubated overnight at 4°C in a 1:500 dilution of anti-Adenovirus serotype 5 polyclonal antibody (Access Biomedical). The cells were then fixed a second time in 4% paraformaldehyde, followed by incubation in CY3-labeled secondary antibody for 1 h. Cells were viewed with a Carv spinning disk confocal (Kinetic Imaging) attached to a Nikon TE300 inverted microscope equipped with a ×60 oil-immersion lens. An image of a single focal plane located at the midpoint of the cell was acquired with a Hamamatsu Orca-ER cooled digital camera using AQM software (Kinetic Imaging). Binding of UV-irradiated and intact adenovirus was quantified by counting the number of puncta with an average size and brightness greater than or equal to 1.2 μm² and 392, respectively, using the AQM spot analysis software. Background level was 200.

Electrophysiological recording of inward currents and membrane capacitance (Cm)

A coverslip was transferred to the recording chamber and continuously perfused with extracellular solution at 2 ml/min. Standard extracellular solution consisted of (in mM) 130 NaCl, 2 KCl, 10 dextrose, 10 Na-HEPES, 1 MgCl₂, 5 CaCl₂, and 5 N-methyl-D-glucamine, pH adjusted to 7.2, 295 mosmol. The calcium channel toxin α-conotoxin GVIA (Bachem America) was stored at ~20°C as a 1 mM stock in 0.1% trifluoroacetic acid and dissolved on the day of experiment in extracellular solution at 1 μM. The toxin was applied to a cell via a large bore capillary tube connected to gravity-fed reservoirs via a four way valve (Warner Instruments). Intracellular solution consisted of (in mM) 135 cesium glutamate, 10 MOPS, 0.5 Na₄BAPTA, and 9.5 NaCl, pH 7.2, mosmol adjusted to 305 with mannitol. Amphotericin B-containing intracellular solution (0.625 mg/ml final concentration) was prepared as described in Thiagarajan et al. (2004). Whole cell patch pipettes were prepared from custom-length micropipette glass (Drummond Scientific) as previously described (Thiagarajan et al. 2004). Experiments were performed at room temperature (20–24°C).

Perforated-patch recordings were initiated when series resistance decreased to 15 MΩ. Series resistance usually stabilized between 8 and 12 MΩ. Although we did not use series resistance correction, it is unlikely that series resistance errors substantially alter our conclusions. We evoke exocytosis with a step to the peak of the current-voltage relationship for calcium currents (~20 mV), so modest shifts in actual voltage due to series resistance errors should not greatly affect the amplitude of the calcium current. In addition, all results are presented as a function of calcium influx, which takes into account any differences in current amplitude due to variable series resistance. Data acquisition was discontinued if holding current was >5 pA. Sodium and calcium currents evoked by depolarization from −90 to +20 mV were fitted with single- or double-exponential functions using Origin (Microcal Software). Data are presented as the mean ± standard error of the mean.
+20 mV were acquired at a sampling rate of 20 kHz for 10- and 40-ms pulses, 5 kHz for 160-ms pulses, and 2.5 kHz for 320-ms pulses and were filtered at 5 kHz. Voltages were not corrected for junction potential.

Phase-tracking capacitance measurements (Fidler and Fernandez 1989) were performed with an Axopatch 200B patch-clamp amplifier (Axon Instruments) controlled by custom Axobasic software running on a PC with a Pentium II 400 MHz processor as described in Thigagaraj et al. (2004). The sine wave stimulus was 1,400 Hz and 40 mV peak to peak. Time resolution was 10 ms per capacitance point. Capacitance measurements were calibrated by triggering a transient addition of 100 pF to the capacitance compensation circuitry.

Carbon fiber amperometry

Amperometric electrodes were prepared from 8-μm-diam carbon fiber (Amoco Performance Products) as described in Thigagaraj et al. (2004). After obtaining a perforated-patch recording, the amperometric electrode was positioned until it was just touching the cell. The electrode was held at +780 mV, and amperometric currents were recorded with a second Axopatch 200B patch-clamp amplifier controlled by custom Axobasic software. Amperometric data were synchronized with capacitance and voltage data by simultaneously acquiring the voltage signal from the patch-clamp amplifier controlling the perforated-patch recording. Data were sampled at 2.5 kHz and filtered at 5 kHz.

Measurements of intracellular calcium

Cells were incubated in 2 μM Fura-4F-AM (Molecular Probes) in Earle’s balanced salt solution (Sigma-Aldrich) for 45–60 min at 37°C. Dual wavelength fluorescence signals were acquired as described in Laflamme and Becker (1996). Briefly, xenon light was passed through a motorized filter wheel that contained transmissive wedges for 340, 380, and 470 nm light. Emitted light was detected by a photomultiplier tube (PMT) attached to a Zeiss Axiovert 10 fluorescence microscope equipped with a ×40 glycerol-immersion lens. Signals were kept in the linear range of the PMT by inserting a neutral density filter in the light path. Background fluorescence at each wavelength was obtained after removing the cell with the patch pipette. In these experiments, bright GFP-positive cells were those with fluorescence at 470 nm that was fourfold greater than the brightest fluorescence of control cells, or >4,000 PMT units. The time resolution was 9 ms per ratiometric measurement. Patch-clamp voltage and inward current were simultaneously acquired at one point for each wedge, each revolution, or 3 ms per point. Capacitance measurements were not performed in cells loaded with Fura-4F.

Data analysis and statistics

Leak current was acquired immediately before each stimulus by hyperpolarizing to −110 mV. Scaled leak current was digitally subtracted prior to integration. Calcium influx in picocoulombs was obtained from integration of the leak-subtracted inward current. Limits were set to exclude the major portion of inward Na+ current. Tetrodotoxin was not included to block Na+ current because it is known to cause a nonexocytotic capacitance increase via slowing of Na+ gating charge movement (Horrigan and Bookman 1994). A nonexocytotic capacitance increase remains in the absence of tetrodotoxin, but it decays faster and is of smaller magnitude.

The capacitance jump was determined by taking the difference between the 200 ms average before and the 200 ms average after the depolarization. Capacitance jumps were corrected for the nonexocytotic capacitance transient by subtracting the average capacitance jumps obtained in a separate set of experiments in which 200 μM cadmium was perfused during 40-, 160-, and 320-ms depolarizations and trains of 10-ms depolarizations. We discarded responses with excess retrieval, a rapid form of endocytosis that may truncate the exocytotic response (Engisch and Nowycky 1998; Smith and Neher 1997). During repetitive stimulation, pulses occurred every 200 ms, so the capacitance jump for one pulse served as the baseline for the next. Therefore the nonexocytotic capacitance increase was only subtracted from the first pulse of a train. A single number for the average efficacy per pulse for pulses 6–30 was obtained for each cell and then averaged to obtain the mean ± SE shown in Fig. 7D (“efficacy”).

Poststimulus drift was taken as the difference between an average of 20 points acquired at the peak of poststimulus capacitance increase and 20 points obtained immediately after the depolarization. The calcium dependence of drift was determined by varying pulse duration and by perfusing cells with solutions containing different concentrations of extracellular calcium (5, 2.5, and 1 mM). For categorizing whether responses showed drift, we chose a threshold of >20 pA. To calculate the amount of drift, when it occurred, all responses with measurable, nonzero drift were included.

Amperometric recordings were performed in a separate set of experiments, so the data in Table 2 and Figs. 4 and 5 are different from the data in Fig. 3. Poststimulus events were counted in the time period starting 200 ms after the end of the depolarization and ending 9 s later. The number of amperometric events that occurred after a depolarization was determined by visual examination of traces displayed at high gain to include slow and small amplitude events that would be missed by an automated peak detection algorithm. Identification of large events was unambiguous; we set a minimum amplitude for small events of 10 pA.

Data were compared with Student’s t-test using Origin (OriginLab). For instances where multiple paired comparisons were made, the Bonferroni correction was applied to determinations of statistical significance. Note that “n” for the same experiment may differ in different figures because not all stimulations were obtained in all cells (single 40-ms depolarization, single 320-ms depolarization, single 160 depolarization, train of 10-ms depolarizations). All error bars are ± SE.

RESULTS

Two days after infection with adenovirus, bovine chromaffin cells displayed a variable amount of fluorescence due to expression of Green Fluorescent Protein (Fig. 1Aii). All data shown have been acquired from “bright” GFP-positive cells. Cells were judged to have bright GFP fluorescence if the appearance were chosen for recordings. Holding current, an indicator of cell damage, was never >5 pA. Therefore the effects we describe are due to viral binding and internalization, reporter expression, or viral protein expression and not to a general loss of cell viability.

Calcium current is reduced in adenovirus-infected cells

Total Ca2+ current was significantly inhibited in adenovirus-infected cells (peak Ca2+ current, control, 644 ± 29 pA; adenovirus, 439 ± 31 pA, P = 0.00001). Figure 1, B and C, shows that adenovirus infection decreases a current that inactivates during a 320-ms depolarization (difference current) more than a plateau current. We have previously demonstrated that the current remaining at the end of a 320-ms depolarization is blocked by ω-agatoxin IVa, a P/Q channel toxin, and the inactivating current is blocked by ω-conotoxin GVLa, an N channel toxin (Engisch and Nowycky 1996; Engisch et al. 1999). To test if N-type current was reduced, we applied 1 μM
ω-conotoxin GVIA to control and adenovirus-infected cells. Figure 1D shows inward currents recorded in a representative control cell and an adenovirus-infected cell before (thick line) and after application of ω-conotoxin GVIA. As expected, the ω-conotoxin-sensitive component is smaller in the adenovirus-infected cell. Furthermore, the peak amplitude after ω-conotoxin was the same in control and adenovirus-infected cells (Fig. 1E), which indicates that adenovirus infection does not inhibit or upregulate any other calcium channel.

The downward spikes that occur immediately after the onset of the depolarization are Na⁺ currents. Na⁺ current amplitudes were determined from the peak inward currents recorded at a faster acquisition rate (not shown). In contrast to Ca²⁺ current, Na⁺ current was significantly larger in adenovirus-infected cells (Table 1).

**Single depolarizations evoke smaller capacitance jumps in adenovirus-infected cells**

Figure 2A shows capacitance responses evoked in a control cell by depolarizations of increasing duration. The amplitude of the capacitance jump is largest for the longest pulse duration. In an adenovirus-infected cell, the responses also increase with pulse duration, but each one is smaller than the corresponding control response (Fig. 2B). Figure 2D shows that the decreases in capacitance responses were of similar magnitude to the decreases in calcium influx, determined from the calcium

![Image](http://jn.physiology.org/)

**FIG. 1.** A variable level of Green Fluorescent Protein (GFP) is expressed in cells infected with GFP-IREs-β-galactosidase adenovirus. Ai: phase contrast image of bovine adrenal chromaffin cells viewed with a ×20 objective at 5 days in vitro. The culture was infected with GFP-IREs-β-galactosidase adenovirus at 3 days in vitro. Aii: same field of view, showing GFP fluorescence. A fraction of the cells expressed high levels of GFP (arrows). Aiii: GFP signal in “bright” GFP-positive cells remained visible when the white light source was on. B: inward currents evoked by 320-ms depolarizations (−90 to +20 mV) in an untreated control cell and in an adenovirus-infected cell that expressed high levels of GFP. C: difference current was reduced in adenovirus-infected cells (**, P < 0.00001). The difference current was obtained by subtracting the current at the end of the depolarization, or the plateau current, from the initial peak current. Adenovirus infection did not affect the plateau current. n = 34 and 40 for control and adenovirus-infected cells, respectively. D: inward currents evoked by 320-ms depolarizations (−90 to +20 mV) in an untreated control cell and an adenovirus-infected cell, before (thick line) and after (thin line) application of 1 μM ω-conotoxin GVIA. E: adenovirus infection selectively reduced the ω-conotoxin-sensitive component of calcium current (*, P = 0.01). n = 9 and 12 for control and adenovirus-infected cells, respectively.

**TABLE 1.** Peak Inward Na⁺ current evoked by a 40-ms depolarization to +20 mV

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Peak Na⁺ Current, pA</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>857 ± 39</td>
<td>39</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>1003 ± 53*</td>
<td>27</td>
</tr>
<tr>
<td>Untreated</td>
<td>621 ± 53</td>
<td>10</td>
</tr>
<tr>
<td>Electroporated</td>
<td>841 ± 84*</td>
<td>11</td>
</tr>
<tr>
<td>Untreated</td>
<td>809 ± 48</td>
<td>21</td>
</tr>
<tr>
<td>UV-irradiated AdV</td>
<td>990 ± 109</td>
<td>14</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>967 ± 73</td>
<td>14</td>
</tr>
</tbody>
</table>

Values are means ± SE. *, P = 0.03 compared to untreated cells. n is the number of cells.
current integral (Fig. 2C). There was no change in the relationship between calcium influx and exocytosis (Fig. 2E). These findings are consistent with previous data showing capacitance jumps are proportional to calcium influx (Engisch and Nowycky 1996; Lukyanetz and Neher 1999; Powell et al. 2000; Ulate et al. 2000).

Unusual capacitance response, poststimulus drift, is observed in adenovirus-infected cells

The capacitance jump measured immediately after the depolarization was not altered in adenovirus-infected cells, but capacitance often continued to increase for several seconds. Figure 3, A and D, shows capacitance measurements that include 5 s after 40-, 160-, and 320-ms depolarizations for a control cell and an adenovirus-infected cell, respectively. The apparently abrupt capacitance jump indicated by the open arrow corresponds to the net increase of capacitance that occurred during the depolarization and is usually taken as the stimulus-evoked response (as in Fig. 2). The maximum poststimulus capacitance increase is indicated by a closed arrow. Control cells rarely showed a poststimulus capacitance increase, and when it occurred, it was small in magnitude (Fig. 3A). In adenovirus-infected cells, poststimulus capacitance increases could reach twice the size of the capacitance jump (Fig. 3D). We refer to the poststimulus capacitance increase as “drift” because it appears to be more gradual than the capacitance jump. Although increased poststimulus drift in adenovirus-infected cells was associated with a decrease in calcium influx, we believe it is a calcium-dependent process for two reasons. First, responses evoked by large amounts of calcium influx were more likely to show drift (Fig. 3E). Second, the amplitude of drift increased with increasing calcium influx (Fig. 3F). In contrast, the small amount of drift observed in control cells showed minimal calcium dependence (Fig. 3, B and C).

Poststimulus exocytosis detected with carbon fiber amperometry is increased in adenovirus-infected cells

Capacitance detection gives the net sum of exo- and endocytosis. Therefore it cannot be used to distinguish whether poststimulus capacitance increases are due to enhanced exocytosis or decreased endocytosis. A carbon fiber electrode placed close to a cell can detect amperometric currents generated by oxidation of catecholamines released on vesicle fusion. If adenovirus only affects endocytosis, the same number of amperometric events should be observed in control and adenovirus-infected cells whether or not we observe a poststimulus capacitance increase.

Cells were depolarized for 160 and 320 ms to maximally induce poststimulus capacitance drift. In Fig. 4, representative capacitance traces (Cm) and simultaneously recorded amperometry traces (Iamp) from control (A) and adenovirus-infected cells (B) are shown.
infected cells (B) demonstrate that increased poststimulus drift was accompanied by a larger number of amperometric events. In both control and adenovirus-infected cells, the number of amperometric events was related to the amount of poststimulus drift (Fig. 5A and Table 2). The average number of amperometric events for control responses with <20 fF of drift was significantly different from 0 (P = 0.00046), which suggests that a small amount of poststimulus exocytosis was missed when capacitance measurements were used for detection. However, the average number of events for drift >49 fF was significantly greater than the number of events for drift <20 fF, in control and adenovirus-infected cells (Fig. 5A). Furthermore, there was a dramatic increase in multiple events in adenovirus-infected cells that showed drift compared with control cells without drift (Fig. 5B). Although we cannot rule out that endocytosis is decreased in adenovirus-infected cells, poststimulus exocytosis is clearly increased.

Global intracellular calcium measurements do not reveal an increase that explains poststimulus drift

Because the trigger for exocytosis is an increase in intracellular calcium, it was surprising to us that in adenovirus-infected cells, the reduction in calcium influx was associated with an increase in exocytosis. Therefore we examined whether another source of calcium might play a role in poststimulus drift. We incubated cells with the membrane permeant AM ester form of Fura-4F and performed dual wavelength ratiometric measurements with a photomultiplier tube as a detector (see METHODS). Figure 6Ai shows the background-subtracted fluorescence ratio measured in a control cell during and after a 320-ms depolarization. The fluorescence ratio rises rapidly during the depolarization then falls exponentially when the voltage pulse is terminated. The fluorescence ratio measured in a bright GFP-positive cell is shown in Fig. 6Aii. For the same pulse duration, the peak rise in global calcium is less than half that of the control cell. The reduction in peak fluorescence ratio is due to the smaller calcium influx; a small calcium response is also observed when the control cell is stimulated with a shorter duration pulse (Fig. 6Aiii). Calcium responses were linearly related to calcium influx, with similar slopes in control and adenovirus-infected cells. (Fig. 6B).

In Fig. 6 it can be seen that the rate of decline of the calcium signal was slower when the calcium signal was small (see insets for extended time course). However, the calcium levels in control cells did not fall below those of the adenovirus-infected cells at 1-s poststimulus (Fig. 6C), a time when poststimulus capacitance increases are robust (Figs. 3D and 4B). Adenovirus binds to integrins on the cell surface; ligand-integrin binding has been shown to induce a calcium transient in some cells (Somogyi et al. 1994; Weismann et al. 1997). Such a rise might lead to over-filling of the readily releasable pool (Voets 2000). There was no difference between control and adenovirus-infected cells in the resting calcium ratio (Fig. 6D). Furthermore, it is unlikely that an increase in [Ca\textsuperscript{2+}] caused the pool to transiently overfill because poststimulus drift could be elicited repeatedly in the same cell (Fig. 3D). In summary, our data show that poststimulus exocytosis in adenovirus-infected cells cannot be attributed to an increase in intracellular calcium concentration.
Cells infected with adenovirus show increased enhancement of exocytosis during stimulus trains

Poststimulus drift is an enhancement of exocytosis that appears to require calcium influx and takes time to develop. Thus it resembles other forms of modulation, including activity-dependent enhancement induced by repetitive stimulation (Engisch et al. 1997; Smith 1999). To test whether activity-dependent enhancement was also increased in adenovirus-infected cells, we stimulated cells with trains of brief depolarizations (Fig. 7). Adenovirus-infected cells showed significantly larger capacitance responses (Fig. 7, A and D) despite smaller calcium currents throughout the train (Fig. 7, A, insets, and C and D). Furthermore, adenovirus-infected cells showed a different pattern of modulation during the train (Fig. 7B, open squares). The capacitance jump showed a less dramatic decline in amplitude during the first several pulses, and amplitude subsequently climbed to a higher value than the initial response. In addition, the average amplitudes after pulse 5 were larger than those of control cells. When the reduced calcium influx was taken into account by dividing each response by the calcium influx for that pulse, adenovirus-infected cells showed a twofold increase in efficacy, in fF/pC, for pulses 6–30 of the train (Fig. 7D).

We examined whether an alteration in global calcium dynamics could explain the paradoxical increase in activity-dependent enhancement in the face of reduced calcium influx. We found that calcium levels reached a lower peak ratio in adenovirus-infected cells (Fig. 8). Although this difference did not reach statistical significance (Fig. 8C, P = 0.14), it is in the wrong direction to account for facilitation. The pulse number at which a plateau occurred was not significantly different between control and adenovirus-infected cells (Fig. 8D, P = 0.4). In summary, we found no evidence that raised global calcium levels were responsible for the activity-dependent enhancement in adenovirus-infected cells.
Modulation of Ca\(^{2+}\) and Na\(^+\) currents may be caused by expression of an exogenous reporter molecule

To test whether expression of an exogenous transgene underlies adenovirus-mediated effects, we used a different method of transfection to introduce a reporter molecule. Nucleofection is a new method of electroporation marketed by Amaxa. We electroporated freshly isolated chromaffin cells using the Nucleofector device in the presence of plasmid DNA for a fluorescent protein from the copepod *Pontellina plumata* ("pMaxGFP") and performed electrophysiological experiments 48 h after plating. In contrast to conventional electroporation, which we previously found induced expression in <5% of cells, nucleofection-induced expression in 30–40% of cells.

Peak calcium current was significantly reduced in electroporated cells (371 ± 36 pA, n = 9 cells) compared with untreated, nonelectroporated cells from the same cultures (556 ± 35 pA, n = 9 cells, P = 0.002). Similar to adenovirus-infected cells, the difference current was most affected (Fig. 9, A and B), but there was also a significant reduction in the plateau current (Fig. 9C). Also similar to adenovirus-infected cells, sodium current was significantly increased (Table 1).

Expression of a reporter molecule does not induce enhancement of exocytosis

Electroporated cells were no more likely than control cells to show poststimulus drift of >20 fF (Table 3). The average amplitude of drift, after discarding responses with 0 fF drift or endocytosis, was similar in the two groups (Table 3). When electroporated cells were stimulated with a train of 10-ms depolarizations to +20 mV, average response amplitude was similar to that of controls throughout the train (Fig. 9D). Calcium influx on the first pulse of the train was reduced in electroporated cells and both groups showed inactivation of calcium influx to a plateau value (Fig. 9E). These results demonstrate that expression of an exogenous reporter does not lead to an upregulation of poststimulus drift, or to an increase in activity-dependent facilitation.

We considered the possibility that the different time in culture was responsible for the lack of effect of nucleofection on poststimulus drift and train-evoked exocytosis. The difference in experimental design—nucleofection at the time of plating (day 0), and adenovirus infection on day 3, was due to the nucleofection requirement that cells be in suspension. The virus infection experiments were performed first before we found that cells did not survive nucleofection after being plated for 3 days and then being resuspended. To rule out this explanation, we performed a set of experiments on cells that were infected with adenovirus on day 0. Average poststimulus drift in cells infected on day 0 was increased compared with drift in control cells from the same cultures (126 ± 30 vs. 16.8 ± 3.6 fF in controls; n = 7 responses from 4 virus-infected cells and n = 5 responses from 3 control cells). Exocytosis during a train of 10-ms pulses was also enhanced in cells infected on day 0. The average efficacy for pulses 6–30 was 7.1 ± 2.1 fF/pC compared with 2.1 ± 0.3 fF/pC in controls. These data indicate it is unlikely the absence of effects in electroporated cells can be explained by the different time in culture.

Probing the mechanism of adenovirally induced potentiation of exocytosis: inactivation of adenoviral DNA

Adenovirus infection begins with adenovirus capsid proteins binding to cell surface receptors, the CAR, coxsackie and adenovirus receptor, and integrins (reviewed in Medina-Kauwe 2003). The role of the capsid can be tested by preventing adenoviral and transgene expression. We exposed the GFP-IREs-β-galactosidase adenovirus to UV light for 30 min in the presence of psoralen, a chemical that binds to viral DNA on UV exposure and blocks virus transcription (Cotten et al. 1992, 1994). To ensure that the capsid was undamaged by this treatment, we infected cells with intact or UV-irradiated virus.

| TABLE 2. Summary of post-stimulus drift responses and amperometric events in cells recorded from with a carbon fiber |
|----------------------------------------------------------|---|---|---|---|---|
| Drift ≤ 20 fF                                           | 41 (72) | 28 | 8 | 4 | 1 |
| Drift 20–49 fF                                          | 9 (16) | 3 | 2 | 1 | 3 |
| Drift > 49 fF                                           | 7 (12) | 0 | 2 | 1 | 4 |
| Total                                                  | 57 | 31 | 12 | 6 | 8 |
| Adenovirus                                             | | | | | |
| Drift ≤ 20 fF                                           | 21 (40) | 18 | 2 | 0 | 1 |
| Drift 20–49 fF                                          | 12 (23) | 2 | 3 | 2 | 5 |
| Drift > 49 fF                                           | 19 (37) | 1 | 6 | 2 | 10 |
| Total                                                  | 52 | 21 | 11 | 4 | 16 |

Each cell was stimulated with a 160-ms and a 320-ms depolarization to +20 mV. The amount of drift was determined as described in METHODS. Responses with excess retrieval were discarded from analysis. An “event” is an amperometric spike recorded with the carbon fiber. Note that compared to previous controls, control cells recorded from with a carbon fiber had a larger number of responses with drift >49 fF. Percentages are enclosed in parentheses.
and 4 h after virus exposure, processed the cells for immunofluorescence with an antibody against adenovirus serotype 5 (Ad5). The 4-h time point was chosen to examine binding and internalization. Figure 10A shows representative confocal images of immunostained cells. In cells infected with the intact or UV-irradiated adenoviruses, immunofluorescence for Ad5 appears as small puncta at the cell surface and within the cell interior, indicating that adenovirus internalization was not impaired by UV irradiation. We counted the number of puncta above a threshold brightness level using an automated spot analysis program (see METHODS). The number ranged from 0 to 6 for cells exposed to the intact virus at the normal concentration, or twice the normal concentration used in physiological experiments (Fig. 10B). We increased the concentration of UV-irradiated virus until the majority of cells displayed multiple puncta (Fig. 10B, “16× UV AdV”). We reasoned that at this dose, cells randomly selected for recording are likely to have as much capsid bound as the bright GFP-positive cells selected in control experiments.

**UV irradiation of adenovirus reduces its effects**

Peak calcium current for cells infected with UV-irradiated virus was 483 ± 35 pA, which was less than that of untreated cells (571 ± 36 pA), but this difference did not reach statistical significance (P = 0.18). The inactivating component of current was reduced in cells infected with UV-irradiated virus, but this difference was also not significant (P = 0.36). Sodium current was increased, but not significantly (Table 1). In the same set of experiments, “positive control” cells that had been infected with intact virus showed a significant decrease in peak calcium current and the inactivating component of calcium current (P = 0.01 and 0.002, respectively). However, the increase in sodium current in cells infected with intact virus also did not reach significance in this experiment (Table 1).

Infection with a UV-irradiated adenovirus caused an increase in the fraction of cells with poststimulus drift that was indistinguishable from the effect of intact adenovirus (Table 3). Although more cells displayed drift, the amplitude of drift was not large (Table 3). The response to repetitive stimulation was in between that of control cells and cells infected with the intact virus (Fig. 10C). Finally, calcium influx for each pulse of the train was reduced, but to a lesser extent than with intact virus (Fig. 10D). Together with the data on calcium and sodium currents, these results show that capsid alone incompletely mimics the actions of the intact adenovirus.

**FIG. 6.** Global [Ca$^{2+}$]$_i$ was not increased in adenovirus-infected cells before, during, or after stimulation with single step depolarizations. A, i–iii: moderate affinity calcium indicator Fura-4F was used to measure changes in global [Ca$^{2+}$]$_i$ evoked by step depolarizations. [Ca$^{2+}$]$_i$ responses are displayed as background subtracted ratios of fluorescence acquired at excitation wavelengths of 340 and 380 nm. The end of the pulse is indicated by the extension of the dashed line from the simultaneously recorded inward current ($I_{in}$). The bottom trace shows the voltage applied to the cell ($V_m$). A: untreated control cell stimulated with a 320-ms depolarization. Aii: adenovirus-infected cell stimulated with a 320-ms depolarization. Aiii: same control cell shown in A, stimulated with a 160-ms depolarization. Insets: declines of fluorescence ratios on an extended time base. B: fluorescence ratio immediately prior to stimulation was subtracted from the peak attained after stimulation, and plotted as a function of calcium influx. The change in fluorescence ratio was a linear function of calcium influx in control (solid) and adenovirus-infected cells (dashes). Control cells, slope, 0.0087 ratio units/pC, $y$ intercept = –0.08 ratio units, $R = 0.64$; adenovirus-infected cells, slope, 0.0092 ratio units/pC, $y$ intercept = –0.09 ratio units, $R = 0.91$. C: there was no difference in fluorescence ratio values at 1 s after the depolarization in control and adenovirus-infected cells. D: resting fluorescence ratio, measured prior to application of any stimulus, was not increased in adenovirus-infected cells. n = 8 control, 7 adenovirus-infected cells.
Interestingly, infection of bovine chromaffin cells with Semliki Forest Virus also caused a reduction in calcium current (Ashery et al. 1999; Duncan et al. 1999; Pan et al. 2002); effects on Na"" currents were not reported. These manipulations have one thing in common, the expression of an exogenous reporter molecule. Overexpression of exogenous proteins may impact a regulatory molecule shared by Ca"""" and Na"" channels that has opposite effects on their function or expression.

Our data show that adenovirus infection increased asynchronous release without altering global calcium dynamics. This result is highly unusual because in chromaffin cells and at fast synapses, the amount of asynchronous release usually parallels the global divalent concentration. For example, asynchronous release is increased in the presence of Ba"""" and Sr"" (Elmqvist and Feldman 1965; Goda and Stevens 1994; Seward et al. 1996). It was recently shown that these divalent reach higher intracellular levels and decay more slowly than [Ca""""]. (Neves et al. 2001; Xu-Friedman and Regehr 1999). Asynchronous release is also increased during and after repetitive stimulation (Delaney and Tank 1994; Elhamdani et al. 1998; Zengel and Sosa 1994). Because asynchronous release appears to be calcium-dependent (see also our Fig. 3), the question becomes: why does it not occur in control cells, which experience higher [Ca""""], levels? Adenovirus infection may remove a barrier to asynchronous exocytosis or dramatically increase its calcium sensitivity.

Li et al. (2002) reported that adenovirus infection did not alter noradrenaline release in bovine chromaffin cells. It is possible that the measurement of radioactively labeled noradrenaline release over a 5-min period sums together the opposite changes in synchronous and asynchronous release, resulting in no net change. Alternatively, the strongly GFP-positive cells we selected for recording may represent a small fraction of the population sampled by release assays.

Adenovirus infection caused an increase in activity-dependent enhancement during repetitive stimulation with brief depolarizations. We have previously reported that activity-dependent enhancement occurs in a third of untreated cells; another third of the cells show activity-dependent depression (Engisch et al. 1997, 1999). Adenovirus infection increased the fraction of cells showing enhancement, to almost 3/4, and appeared to eliminate depression. The increase in facilitation is calcium independent in the sense that it is not the result of higher [Ca""""], during stimulation. Although we have previously shown that lower calcium influx can lead to an increase in the likelihood of facilitation, this occurred when calcium influx was decreased many-fold, for example, when pulse duration was decreased from 40 to 10 ms (Engisch et al. 1997). In the present study, calcium influx in control cells was already small enough to maximize the probability of facilitation. Indeed, when we compared control cells that had the smallest calcium currents with those that had the largest, the former did not show greater facilitation (data not shown).

Poststimulus exocytosis and activity-dependent enhancement were not increased in electroporated cells. This result further supports the conclusion that a decrease in calcium influx of 30–40% does not by itself lead to increased facilitation. In addition, the electroporation results demonstrate that overexpression of a reporter molecule does not cause enhancement of exocytosis. We do not think the differences between adenovirus infection and nucleofection can be attributed to the
shorter time in culture for nucleofected cells because a small group of cells infected with adenovirus on the day of plating and examined on day 2 of culture showed robust facilitation. Because the reporters in the electroporation and adenovirus experiments were not identical, it remains a possibility that the alterations in exocytosis were caused by the particular type of GFP produced by our adenovirus or by the β-galactosidase. However, the increases in poststimulus and train-evoked exocytosis were also observed in adenovirus-infected cells expressing an enhanced GFP-tagged protein, without β-galactosidase (Thiagarajan et al. 2004). In addition, UV irradiation of the adenovirus, which completely prevented expression of GFP and β-galactosidase, only partially inhibited the effects on poststimulus and activity-dependent exocytosis.

We tested UV-irradiated virus to determine if adenovirus effects could be completely explained by capsid interaction with cell surface receptors. UV irradiation clearly reduced the effectiveness of the virus, therefore capsid binding alone cannot explain our results. Although we cannot rule out that less asynchronous release and activity-dependent facilitation were due to fewer capsid binding events, this is unlikely because we used high concentrations of irradiated virus, that caused multiple surface binding events. We conclude that expression of viral genes causes enhancement of exocytosis. “Leaky” expres-

![FIG. 8.](image1)

**FIG. 8.** Global \([\text{Ca}^{2+}]_i\) was not increased in adenovirus-infected cells during or following repetitive stimulation. \(A\) and \(B\): fluorescence ratio measurements performed with Fura-4F during repetitive stimulation with 10-ms depolarizations to \(+20\) mV from a holding potential of \(-90\) mV, in a control cell \((A)\) and an adenovirus-infected cell \((B)\). Below each ratio measurement is the voltage-clamp current recording, which shows calcium current amplitude throughout the train \((I_m)\). The onset of the plateau \((- - -)\) was determined by the point at which the ratio trace deviated from a straight line aligned to the early part of the response \((\cdot \cdot \cdot)\). \(C\): plateau value was taken as the peak ratio. The peak ratio was slightly smaller in adenovirus-infected cells, but this difference was not significant. \(D\): pulse number at which the plateau occurred was indistinguishable in control and adenovirus-infected cells.

![FIG. 9.](image2)

**FIG. 9.** Electroporation of a plasmid encoding an exogenous reporter causes a decrease in calcium currents but does not alter activity-dependent modulation during repetitive stimulation. \(A\): Representative inward currents evoked by a 320 ms depolarization to \(+20\) mV from a holding potential of \(-90\) mV in a control cell and an electroporated cell expressing pMaxGFP. \(B\): the difference current, determined by subtracting the plateau current from the peak current, was significantly reduced in electroporated cells *(, \(P = 0.013)\). \(C\): the plateau current was significantly reduced in electroporated cells (**, \(P = 0.0047\)). \(D\): average capacitance jump for each pulse of a train of thirty 10 ms depolarizations to \(+20\) mV from a holding potential of \(-90\) mV, 200 ms between depolarizations. \(n = 8\) for untreated control cells, \(9\) for electroporated cells. \(E\): average calcium influx, determined by integrating the calcium current, for each pulse of the train in control and electroporated cells.
cytoskeleton (reviewed in Miranti and Brugge 2002), which have been implicated in the modulation of calcium-evoked exocytosis (Gillis et al. 1996; Li et al. 2003; Smith 1999; Vitale et al. 1995).

Our results suggest that adenovirus infection does not affect the size or calcium sensitivity of the immediately releasable or the readily releasable pools. It did not increase exocytosis evoked by single 10-ms depolarizations that occurs from the immediately releasable pool or exocytosis evoked by depolarizations ≤320 ms that occurs from the readily releasable pool (Voets et al. 1999). A slowly releasable pool was first described as a late component in response to flash photolysis of caged calcium (Heinemann et al. 1994). In mouse chromaffin cells, the slowly releasable pool is not released by depolarization, only by flash photolysis (Voets 2000; Voets et al. 1999). Adenovirus infection may alter the kinetics or calcium sensitivity of the slowly releasable pool, or, remove a barrier, so that it is now able to fuse after depolarizations. Although we did not confirm the identity of the adenovirus-sensitive pool with flash experiments (they are not possible in perforated patch mode), the importance of our findings is that adenovirus infection increases fusion from a pool that is distinct from the immediately releasable and readily releasable pools.

While the effects of adenovirus could be considered bothersome artifacts, we can exploit them to gain insight into the properties of exocytosis and its modulation. Presumably adenovirus acts by disrupting the function of cellular proteins. For example, adenovirus infection alters the localization of a Rab3 family member, Rab3D, in lacrimal gland cells (Wang et al. 2000).

### Table 3

| Treatment                  | Cells With Poststimulus Drift > 20 fF | Amount of Poststimulus Drift (fF) | No. of Responses
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>3/9 (33%)</td>
<td>39 ± 10</td>
<td>5</td>
</tr>
<tr>
<td>Electroporated</td>
<td>3/8 (37.5%)</td>
<td>31 ± 3</td>
<td>5</td>
</tr>
<tr>
<td>Untreated</td>
<td>5/17 (29%)</td>
<td>26 ± 7</td>
<td>19</td>
</tr>
<tr>
<td>UV-irradiated AdV</td>
<td>7/11 (64%)</td>
<td>44 ± 9</td>
<td>11</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>8/11 (73%)</td>
<td>67 ± 14*</td>
<td>16</td>
</tr>
</tbody>
</table>

Each cell was stimulated with a 160-ms and a 320-ms depolarization to +20 mV. The amount of drift was determined as described in Methods. Responses with excess retrieval were discarded from analysis. *, P = 0.016 compared to untreated cells (after Bonferroni correction for 2 comparisons). †All responses with >0 fF drift were included in calculation of amount of drift.

---

**FIG. 10.** UV-irradiated adenovirus can bind to chromaffin cells and be internalized but is less effective than intact virus at causing increased exocytosis during repetitive stimulation. A: adenovirus serotype 5 immunoreactivity in an untreated chromaffin cell, a cell infected with intact adenovirus (2× AdV), and a cell infected with UV-irradiated virus (16× UV-Adv). Cells were processed for immunofluorescence 4 h after exposure to virus. Confocal images of the equatorial plane were obtained with a spinning disk confocal attachment (Carv). The 2× and 16× refer to the relative concentration of virus applied to cells, compared with the concentration used in physiological experiments. Scale bar: 10 μm. B: puncta were counted by an automated spot detection software (see Methods). Each data point (○) corresponds to a single confocal image; 1 image was analyzed per cell. C: average capacitance jump amplitude for each pulse of a train of 30 10-ms depolarizations, −90 to +20 mV, 200 ms between pulses, in control untreated cells (n = 17), cells infected with UV-irradiated adenovirus (n = 13), and cells infected with intact adenovirus (n = 13). D: calcium influx, determined from the integral of calcium current for each pulse of the train in control cells, cells infected with UV-irradiated adenovirus, and cells infected with intact adenovirus.
2003, 2004). Interestingly, this effect was still present when the virus was inactivated by UV-irradiation, suggesting it is mediated by the binding of capsid proteins to cell surface receptors. We recently showed that the expression of Rab3A, and an activated Rab3A mutant, abolished poststimulus exocytosis and activity-dependent enhancement in adenovirus-infected chromaffin cells (Thiagarajan et al. 2004). It will be interesting to determine the expression level and localization of Rab3A in chromaffin cells infected with control and UV-irradiated adenoviruses.

Current studies of neuronal gene therapy using adenovirus are focused on promoting the survival of neurons (Chmielnicki et al. 2004; Nakaizumi et al. 2004). When adequate survival is achieved, the function of the rescued neurons will become important. Adenovirus infection alters regulated secretion in multiple cell types, including lacrimal acinar cells (see preceding text), pancreatic acinar cells (at high levels) (Padfield et al. 1998), and bovine chromaffin cells (our study). It will be important to determine if adenovirus affects synaptic release of neurotransmitter from a fast synapse. Our data are the first to show that in a model secretory cell, adenovirus infection causes a shift from synchronous release to asynchronous release, and a calcium-independent enhancement of facilitation. If these changes occurred in the CNS, information transfer would be profoundly altered. In this light, it is notable that behavioral changes have been observed after injection of a control adenovirus into the brain (Bilang-Bleuel et al. 1997).

Our results suggest caution in using adenovirus either as a vector for exogenous expression studies or for gene therapy in the CNS. On the bright side, the upregulation of poststimulus exocytosis and activity-dependent exocytosis by adenovirus infection has allowed us to begin probing the mechanisms of these elusive behaviors. In the future, adenovirus might be exploited for its ability to enhance neurotransmitter release.

Acknowledgments

We thank P. Becker for advice and assistance with measurements of global calcium. We are grateful to L. Medina-Kauwe for helpful discussions and S. Hamm-Alvarez for valuable comments on an earlier draft of the manuscript. Present address of R. Thiagarajan and M. M. Rich: Dept. of Neuroscience, Cell Biology, and Physiology, Wright State University, Dayton, OH 45435.

Grants

This work was supported by National Institute of Mental Health Grant MH-64744.

References


Gillis KD, Mossier R, and Neher E. Protein kinase C enhances exocytosis from chromaffin cells by increasing the size of the readily releasable pool of secretory granules. Neuron 16: 1209–1220, 1996.


