Efficient transfection of dissociated mouse chromaffin cells using small-volume electroporation

Widmann W. Hoerauf · Victor A. Cazares ·
Arasakumar Subramani · Edward L. Stuenkel

Received: 14 August 2013 / Accepted: 1 February 2014 / Published online: 19 February 2014
© Springer Science+Business Media Dordrecht 2014

Abstract We have developed an improved procedure for isolating and transfecting a chromaffin cell-enriched population of primary cells from adult mouse adrenal glands. Significantly, the parameters of a novel electroporation transfection technique were optimized to achieve an average transfection efficiency of 45 % on the small number of cells derived from the mouse glands. Such transfection efficiency was previously unachievable with the electroporation protocols conventionally used with bovine chromaffin cells, even with use of large cell numbers. Our small scale technique now makes feasible the use of genetically homogenous inbred mouse models for investigations on the exocytotic pathway without the time, expense, and cellular changes associated with viral approaches. High fidelity co-expression of multiple plasmids in individual cells is a further advantage of the procedure. To assess whether the biophysical characteristics of mouse adrenal chromaf-

fin cells were altered by this process, we examined structural integrity using immunocytochemistry and functional response to stimuli using calcium imaging, amperometry, and whole-cell capacitance and current clamp recordings. We conclude these parameters are minimally affected. Finally, we demonstrate that high transfection efficiency makes possible the use of primary mouse adrenal chromaffin cells, rather than a cell line, in human growth hormone secretion assays for high throughput evaluation of secretion.

Keywords Mouse · Chromaffin ·
Dissociation · Electroporation · Transfection ·
hGH secretion assay

Introduction

The regulated exocytotic release of chemical messengers provides the primary mechanism for information transfer within the central nervous and endocrine systems. Extensive studies investigating the molecular mechanism and kinetic parameters of regulated exocytosis have principally utilized chromaffin cells of the adrenal medulla. These neuroendocrine cells which functionally resemble postganglionic neurons release catecholamine neurotransmitters into systemic circulation in response to splanchnic nerve sympathetic input.

W. W. Hoerauf (✉) · V. A. Cazares · A. Subramani ·
E. L. Stuenkel
Department of Molecular and Integrative Physiology,
University of Michigan, 1150 W. Medical Center Dr.,
Ann Arbor, MI 48109-0622, USA
e-mail: whoerauf@umich.edu

W. W. Hoerauf · V. A. Cazares · E. L. Stuenkel
Neuroscience Graduate Program, University of Michigan,
4137 Undergraduate Science Building, 204 Washtenaw
Ave., Ann Arbor, MI 48109-2215, USA

Chromaffin cells possess multiple advantages for studying exocytosis. First, they utilize a complement of exocytotic proteins similar to those found in other endocrine cell types and the presynaptic nerve endings of neurons, though chromaffin cells lack morphologically distinct active zones. Therefore, functional effects manifest by perturbation of chromaffin cell protein expression has greatly informed our knowledge of the regulated exocytotic mechanisms of all cell types. Second, chromaffin cells enable millisecond resolution of evoked vesicle fusion kinetics through application of carbon fiber amperometry and membrane capacitance monitoring, facilitating the identification of functionally distinct vesicle pools and essential exocytotic pathway protein–protein interactions. Third, dense-core vesicles are large enough that, using total internal reflection fluorescence microscopy (TIRFM) with fluorescently tagged vesicle proteins, granule recruitment to, and mobility at, plasma membrane sites of exocytosis can be analytically evaluated and individual fusion events directly examined. Finally, due to the extensive use of chromaffin cells by numerous labs over decades of research, a wealth of previously published data exists that characterizes a broad range of biophysical metrics and affords already-refined methods of electrophysiological data analysis. For these reasons, chromaffin cells continue to be an exceptional model for studying molecular exocytotic mechanisms.

Common approaches for overexpression and knockdown of protein expression include introduction of nucleotide sequence via viral infection or calcium phosphate transfection. Semliki Forest virus (SFV), closely related to Sindbis virus (Ehrensgruber et al. 2011), has been used since 1999 to efficiently transduce (Duncan et al. 2002) bovine chromaffin cells (Ashery et al. 1999; Duncan et al. 1999). However, SFV transduced cells exhibit cell morphology changes, possess a smaller readily releasable pool, and exhibit reduced calcium currents (Ashery et al. 1999). Infected cells also demonstrate limited endogenous transcription/translation, synthesizing viral proteins instead. Adenovirus can also achieve high transduction efficiency (Li et al. 2002), though it alters cell structural and functional integrity (Alesci et al. 2007) with profound nuclear and mitochondrial changes and a decrease in cell survival and NE uptake that is time- and dose-dependent. Limiting to viral strategies is the time and cost required for recombinant

viral production. Calcium phosphate transfection of mammalian cells is perhaps the most established and commonly used technique (Graham and van der Eb 1973). Although many modifications to the basic procedure exist (Jordan and Wurm 2004; Wilson and Smith 1997) most investigations report transfection efficiencies of 0.5–1 % with bovine chromaffin cells, an unacceptably low value when applied to the small number of chromaffin cells present in the mouse adrenal medulla.

Since first proposed (Alesci et al. 2007; Graham et al. 2000), electroporation has become a potent and useful tool mediating prokaryote plasmid transduction and efficient eukaryote transfection. Unfortunately, its conventionally large cell number requirement has precluded its use when limited tissue mass, as with mouse adrenal glands, results in few isolated primary cells. Here we optimize use of a new commercially available capillary electroporation system (Neon Transfection System, Life Technologies) for transfection of small numbers of mouse chromaffin cells. Using the capillary system, cell density is kept high by resuspending cells in 10 μ L of buffer per electroporation, a volume in which the cells will be electroporated.

Mouse adrenal chromaffin cells offer significant advantages over chromaffin cells of bovine origin. Established mouse strains provide a known and uniform genetic background, are readily available from both commercial breeders and academic institutions, and can be bred and maintained in-house. Bovine material, on the other hand, must be collected from off-site abattoirs and is genetically inconsistent. Significantly, there also exists a diverse and ever growing assortment of genetic mouse models available to the specific needs of the investigator. With these benefits in mind, this study's goal was to develop an improved method for efficient isolation and transfection of mouse adrenal chromaffin cells and thereby facilitate investigation of the mechanisms of specific secretory pathway molecules.

Materials and methods

Plasmids

Neuropeptide Y-mCherry (Anantharam et al. 2011), pEGFP–C1 (Clontech, Mountain View, CA, USA;

GenBank Accession # U55763), and human growth hormone (hGH) gene subcloned into pXGH5 (Wick et al. 1993) were used in this study.

Mice

C3H/HeSnJ mice (Jackson Labs, Bar Harbor, ME, USA) were bred on-site. All experiments were carried out according to rules and regulations of the University Committee on Use and Care of Animals (UCUCA).

Chromaffin cell dissociation

Female mice (9–12 week-old) were euthanized through carbon dioxide asphyxiation, followed by decapitation. Excised adrenal glands were placed in 4 °C physiological saline solution (PSS; in mM: 80 Na-glutamate, 55 NaCl, 6 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, 10 glucose, pH 7.0, 315 mosmol L⁻¹). Following adipose and capsule removal, glands were embedded in low melting point agarose (Gibco 15517-014; Life Technologies, Carlsbad, CA, USA) and sectioned at 200 µm in 4 °C nominal-CaCl₂ PSS (PSS-Ca²⁺) using a vibratome (Ted Pella Incorporated, Redding, CA, USA). Agarose and the cortical region were trimmed away in 4 °C PSS, generating isolated medullary fragments. Cells used for electroporation were isolated from medullary fragments held overnight on membrane inserts (Millicell PIC-MORG50; Millipore, Billerica, MA, USA) over DMEM (Gibco 11960) containing 1 % ITSX (Gibco 1101), 1 % Glutamax (Invitrogen 35050-061), and pen/strep (Gibco 15070-063).

Medullary fragments were rinsed with 5 mL Ca²⁺/Mg²⁺-free PBS (PBS) and digested (37 °C, 10 min) with 1 mL of PSS-Ca²⁺ containing 30 U/mL papain (Worthington Biochemical, Lakewood, NJ, USA), 150 µM DTT, and 0.5 mg/mL BSA (A7906; Sigma-Aldrich, St. Louis, MO, USA). The enzyme solution was removed and replaced with 1 mL of PSS-Ca²⁺ containing 3 U/mL collagenase F (Sigma C7926), 0.5 mg/mL BSA, and 100 µM CaCl₂ (37 °C, 10 min). Fragments were rinsed with PBS, if the cells were to be electroporated, or 4.5 mL F-12 K medium (Gibco 21127) supplemented with 15 % horse serum (Gibco 16050-130) and 2.5 % fetal bovine serum (Gibco 10437-028), if they were not. Fragments were finally resuspended in 1 mL of PBS, or the supplemented

F-12 K medium, and triturated 10× using a 1 mL pipette tip and then 20× using a 200 µL Precise Point Neptune tip (2102Y; Neptune, San Diego, CA, USA) mounted to the distal end of the 1 mL tip. Dissociated cells were either subjected to electroporation or plated onto poly-D-lysine (70–150 kD, Sigma P6407) coated 25 mm round glass coverslip-bottomed 35 mm culture dishes. Plated cells were allowed to settle before addition of medium. Cells were held in culture at 37 °C in 10 % CO₂ for 2 days post-transfection before experimental measurements were made but could be cultured for up to 5 days.

Chromaffin cell electroporation

Dissociated cells were centrifuged for 6 min at 400×g. The supernatant was removed and the cell pellet resuspended at a ratio of 30,000 cells/10 µL of the proprietary resuspension buffer provided with the Neon Transfection kit (Life Technologies). Endotoxin-free plasmid containing cDNA inserts (Endo-Free Plasmid Maxi Kit, Qiagen, Hilden, Germany) was added and gently mixed with the cells. To each 10 µL electroporation, one voltage pulse was applied for 20 ms. Electroporated cells were expelled into F-12 K medium (Gibco 21127), supplemented with 15 % horse serum (Gibco 16050-130) and 2.5 % fetal bovine serum (Gibco 10437-028), and plated onto coverslips, as described above.

Transfection efficiency and viability

Transfection efficiency and viability were assessed by EGFP expression and Trypan Blue exclusion, respectively, 2 days following electroporation with 0.2 µg of EGFP plasmid per electroporation. A cell was considered transfected if the average intensity (background-subtracted for autofluorescence at that wavelength) of a region of interest on a cell was at least twice the average intensity of cells in a non-transfected control group. MetaMorph (Molecular Devices, Sunnyvale, CA) software was used for image analysis. Briefly, cells were rinsed 2× and placed in PSS containing 0.2 % Trypan Blue Stain (Gibco 15250-061). Images were acquired using an Olympus IX81 microscope, 20× objective, 2× expander, EGFP (Ex 472/30 nm, Em 520/35 nm) and mCherry (Ex 562/40 nm, Em 641/75 nm) filter sets, and an ImageEM camera (Hamamatsu, Bridgewater, NJ, USA).

For cotransfection experiments, 0.2 µg EGFP and 0.2 µg NPY-mCherry plasmids were used and images acquired 2 days following transfection. Cells were considered successfully transfected by EGFP and/or NPY-mCherry using the abovementioned criterion. Cotransfection efficiency was calculated based on the relative intensity of fluorescence from each protein.

Immunocytochemistry

Cells were rinsed 1× with PBS before 4 % paraformaldehyde/4 % sucrose fixation (4 °C, 10 min), and 0.1 M glycine quenching (5 min). Cells were rinsed 1× with PBS, permeabilized with 0.25 % Triton X-100 (8 min), then blocked with 10 % BSA in PBS (1 h). Cells were incubated with primary antibody in PBS (3 % BSA) (1 h), rinsed with PBS (5×, 5 min), then incubated for 45 min with secondary antibody. Cells were rinsed (5×, 5 min) with PBS and covered in 70 % glycerol in PBS. When used, 300 nM DAPI was included in the second rinse. Reagents include Alexa Fluor 568 phalloidin (Molecular Probes, A12380; Life Technologies), DAPI FluoroPure (Molecular Probes, D21490), dopamine β-hydroxylase Ab (provided by R. Holz, Ann Arbor, MI), Calnexin pAb (ADI-SPA-860; Enzo Life Sciences, Farmingdale, NY, USA), panVAMP Ab (104 102; Synaptic Systems, Göttingen, Germany), Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes A-11029), and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes A-11034). Cells were imaged by spinning disc confocal microscopy (IX81, DSU; Olympus, Center Valley, PA, USA) or TIRFM (Axelrod 1981) for panVAMP antibody.

Ratiometric calcium imaging

Cells were loaded with 2 µM Fura-2 AM (Invitrogen F1221) (37 °C, 12 min), rinsed 2× with PSS, and the coverslip mounted in a low-profile perfusion chamber (RC-49FS; Warner Instruments, Hamden, CT, USA). Fluorescence emission images (520 ± 35 nm) of cells were acquired at 4 Hz in response to alternating 340 ± 10 and 380 ± 10 nm excitation using an Olympus UApo/340 20× objective (0.75 N.A.) on an Olympus IX81 microscope under control of MetaFluor Ratio Imaging Software (Molecular Devices, Sunnyvale, CA, USA). Intracellular $[Ca^{2+}]$

was reflected in the fluorescence emission intensity ratio corresponding to 340 nm/380 nm excitation.

Electrophysiology

Membrane capacitance was monitored using an EPC9 amplifier (HEKA Elektronik, Lambrecht, Germany) with Patchmaster Lock-in extension (sine + dc) and 2,500 Hz 70 mV sinusoid wave. Bath solution contained (in mM): 137 TEA-Cl, 2 CaCl₂, 2 MgCl₂, 10 Na-HEPES (pH 7.4), and 19 glucose. Pipette solution contained (in mM): 145 Cs-glutamate, 8 NaCl, 1 MgCl₂, 2 Mg-ATP, 0.3 Na₂-GTP, 10 Cs-HEPES (pH 7.2), and 0.1 EGTA. Pipettes (2–4 MΩ) pulled from borosilicate glass were coated with Sylgard (Dow Corning, Midland, MI, USA). Whole-cell voltage clamped cells were held at −80 mV and six 10 ms pulses to 0 mV at 3 Hz, followed by six 100 ms pulses at 2.5 Hz were applied to induce Ca²⁺ influx and exocytotic activity. Patcher's Power Tools (www.mpibpc.mpg.de/groups/neher) running within Igor (Wavemetrics, Lake Oswego, OR, USA) was used for analysis.

Whole-cell current clamp was used to determine action potential thresholds. For these measurements current was initially injected to maintain cells at −80 mV, then 2 s depolarizing-step injections were applied that progressively increased by 1 pA per trace until action potentials were first observed. The inter-injection interval was 9 s. External solution contained (in mM): 119 NaCl, 2.5 KCl, 2 CaCl₂, 2 MgCl₂, 25 Na-HEPES (pH 7.4), and 30 glucose. Pipette solution contained (in mM): 135 K-gluconate, 7 NaCl, 2 MgCl₂, 2 Na-ATP, 0.3 GTP, 10 Na-HEPES (pH 7.2), and 0.2 EGTA.

Electrochemical amperometric recordings employed 5 µm carbon fiber electrodes (CFE) (ALA, Farmingdale, NY, USA), as previously described (Barclay et al. 2003; Graham et al. 2000). Cells were stimulated by perfusion for 2 min with 75 mM K⁺ in Na⁺-reduced (by 70 mM) PSS. Amperometric current was recorded using an EPC-9 amplifier and a CFE charged to 700 mV. Data sampled at 10 kHz and filtered at 2.9 kHz were analyzed with an Igor quantal release XOP (www.sulzerlab.org). Only spike currents >20 pA, and 25–75 % rise times <2 ms, were analyzed. Currents were considered pre-spike feet only when duration exceeded 1 ms.

Regulated secretion assay

Human growth hormone (hGH) secretion from transfected mouse chromaffin cells was quantitatively determined using an enzyme immunoassay (Roche Applied Science, Indianapolis, IN, USA), as previously described (Wick et al. 1993). Briefly, cells were electroporated with hGH plasmid and seeded in 24-well plates (Costar 3524; Corning Incorporated, Corning, NY, USA) at a density of $\sim 10,000$ cells/well. After 2 days, cells were washed with PSS (PSS; in mM: 145 NaCl, 5.6 KCl, 0.5 MgCl_2 , 2.2 CaCl_2 , 15 HEPES, 2.5 glucose, 2 mg/ml Na-ascorbate, pH 7.4) and incubated with either low (5.6 mM K^+) or elevated KCl (56 mM K^+ ; Na^+ reduced to maintain tonicity) PSS (37 °C, 10 min). The PSS was collected to determine secreted hGH, while cell lysates were used to determine total hGH content. Cells were lysed in 0.2 mM EDTA, 10 mM HEPES, pH 7.4, 1 % Triton X-100 (w/v), with the lysate briefly sonicated and centrifuged at $400\times g$ for 5 min.

Results and discussion

To prepare purified adrenal chromaffin cells, intact adrenal glands are sliced into thin sections (Fig. 1a) of identical thickness to ensure uniform digestive enzyme access. In addition, the surrounding cortical tissue is precisely removed from the inner medulla prior to enzyme treatment, resulting in few hormone-secreting cortical cells following dissociation (Fig. 1b, c). That the dissociated cells were enriched highly in chromaffin cells was demonstrated by dopamine β -hydroxylase ICC where 91.7 % of cells were positive for this chromaffin cell-specific marker in adrenal

tissue (Aunis et al. 1980). By comparison, existing protocols use scissors for cortex removal from intact glands, which is considerably less effective, or and pull apart the medulla with forceps prior to digestion. Our slice trimming procedure is less disruptive and largely eliminates cortical cell contamination. We found adult male and female mice yield $\sim 25,000$ and $\sim 40,000$ dissociated cells chromaffin per animal, respectively.

Membrane permeabilization during electroporation occurs only where the electrically induced potential difference between the inside and outside of the cell reaches a critical value of ~ 200 mV (Kotnik and Miklavcic 2000), irrespective of cell type (Teissie and Rols 1993); however, the critical field intensity necessary to achieve that value depends on cell size and must be optimized for each cell type (Rols and Teissie 1990). Passage of DNA through the cytoplasm and into the nucleus is poorly understood but does not depend on the electric field (Kee et al. 2011). To develop an optimized protocol, 30,000 cells were electroporated with EGFP plasmid. Transfection efficiency was quantified by EGFP expression, while viability was simultaneously evaluated using Trypan blue exclusion. Bright field and corresponding fluorescent images (Fig. 2a) illustrate the three possible outcomes. Cells may be *transfected and viable* (white arrow; expressing EGFP, excluding Trypan blue), *non-viable* (black arrow; Trypan blue positive), or *non-transfected and viable* (red arrow; no GFP, excluding Trypan blue). As applied electroporation voltage is increased, the percentage of viable cells decreases (Fig. 2b). Maximum transfection efficiency was 45 % at 1,400 V (Fig. 2c), a voltage that retained 70 % viability. By comparison, standard 4 mm cuvette electroporation of bovine chromaffin cells

Fig. 1 Isolation of mouse adrenal chromaffin cells. **a** Bright-field image of a 200 μm thick slice of isolated adrenal gland ($\times 20$). **b** Differential interference contrast (DIC) image of

isolated chromaffin cells. **c** DIC image of a single isolated chromaffin cell ($\times 60$). Scale 10 μm

Fig. 2 Electroporation-mediated transfection of isolated mouse chromaffin cells. **a** Brightfield and fluorescent image pair demonstrating transfected (EGFP positive, *white arrow*), non-transfected (*red arrow*), and non-viable (Trypan blue positive, *black arrow*) chromaffin cells. **b** Histogram relating percentage of viable chromaffin cells to electroporation voltage. **c** Histogram relating percentage of transfected cells to electroporation voltage. **d** Brightfield (*left*) and fluorescent images (*right*) demonstrating efficient co-transfection of EGFP

and NPY-mCherry plasmids. **e** Histogram illustrating cotransfection efficiency of chromaffin cells electroporated with both EGFP and NPY-mCherry plasmids. **f** Scatter plot of individual background-subtracted chromaffin cell EGFP and NPY-mCherry intensities normalized to maximum intensity (F/F_{max}) in cells cotransfected with both plasmids. *Line* is a least squares fit of the data. Results are $ave. \pm SEM$. Number (N) of separate experiments: **b** and **c**, N = 3; **e** and **f**, N = 2. (Color figure online)

requires 10^7 cells while achieving only 2–5 % transfection efficiency (Graham et al. 2000). Electroporations for all experiments below were done at 1,400 V.

We next determined the extent to which the developed electroporation method allows co-transfection of individual electroporated cells with multiple plasmids. For these experiments chromaffin cells were electroporated with both EGFP- and mCherry-expressing plasmids. Figure 2d shows a DIC bright field image, and corresponding fluorescence images, of electroporated cells, with four cells co-expressing EGFP and mCherry. For cells expressing EGFP we then measured its background corrected average fluorescence intensity and determined if it also expressed NPY-mCherry by measuring its averaged fluorescence and vice versa. Figure 2e shows the results of plasmid co-expression. Of those cells expressing one plasmid, 97 % also expressed the

other. The extent of co-expression among transfected cells is illustrated in a scatter plot (Fig 2f), where each axis is normalized to the highest average fluorescence intensity for that protein among the cells measured. The dynamic range of expression levels following background subtraction extends over two orders of magnitude. A least squares fit indicates linear correlation of EGFP and mCherry protein expression. The importance of this finding is that by visualizing expression of a fluorophore expressed from a “reporter” plasmid, one can assume the expression of a separate plasmid, if those two plasmids are co-transfected.

An important attribute of any transfection technique is the method must not significantly impact the biological process under investigation. To assess possible gross structural changes, specific cellular compartments were examined using immunocytochemistry in

Fig. 3 Normal chromaffin cell organelle morphology maintained following electroporation protocol. Treatments compared include: cells exposed to electroporation protocol but not receiving voltage pulse, cells receiving voltage pulse but not transfected, and cells voltage pulsed and transfected with EGFP plasmid

successfully (GFP-positive) and unsuccessfully (no fluorescence) electroporated cells, in addition to cells exposed to identical handling but no electroporation voltage pulse. No differences were discernible among the three conditions in images of endoplasmic reticulum, secretory granules, cortical actin, or the nucleus when these compartments were labeled with calnexin or panVAMP antibodies, phalloidin, or DAPI staining, respectively (Fig. 3), indicating normal cell morphology and subcellular compartmentalization was maintained.

Regulated secretion is initiated by a stimulus-induced increase in intracellular Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$. Using the fluorescent Ca^{2+} indicator Fura2, we determined whether the electroporation procedure alters the ability of chromaffin cells to increase $[\text{Ca}^{2+}]_i$ in response to a 10 s stimulation with PSS containing

56 mM K^+ or 10 μM DMPP (dimethylphenylpiperazinium), a nicotinic acetylcholine receptor (nAChR) agonist. Comparison of average fluorescence ratios (Fig. 4) revealed no differences with basal (gray bars) or stimulation (black bars) PSS under all conditions, except for a significantly higher calcium increase with freshly dissociated cells. These results indicate successful electroporation does not itself disturb calcium signaling and demonstrates that nAChRs are present and functional 2 days following chromaffin cell dissociation.

Particular attention was also given to whether the electroporation procedure alters cell secretory properties. To provide a highly resolved temporal analysis of secretory events, and to quantify specifically defined pools of secretory granules, we employed membrane capacitance monitoring of exocytotic

Fig. 4 Effect of electroporation protocol on stimulus-evoked changes in $[Ca^{2+}]_i$ in chromaffin cells. **a, b** Relative $[Ca^{2+}]_i$ of chromaffin cells held in 5.6 mM K^+ PSS (basal, light bars) or perfused with **a** 56 mM K^+ PSS (stimulated, dark bars) or **b** 10 μ M DMPP PSS (stimulated, dark bars) as reported by the fluorescent Ca^{2+} indicator Fura2. Histograms show emission intensity ratios corresponding to 340/380 excitation for cells that

were either freshly dissociated (Diss), processed up to electroporation (Non-Pulsed), or electroporated (Pulsed and Transfected). Experiments were performed twice, except for the dissociated condition, which was done once. Number of cells imaged is indicated above each bar. *** $p < 0.0001$, two-tailed Student's t test, equal variance. Results are ave. $\pm$ SEM

fusion events evoked by membrane depolarizations applied under whole-cell voltage clamp. Administration of six 10 ms depolarizing pulses drives fusion of granules in close proximity to calcium channels, the immediately releasable pool (IRP) (Voets et al. 1999), with pool depletion evidenced by a plateau in membrane capacitance (ΔC_m). An immediate subsequent administration of six 100 ms depolarizing pulses results in additional calcium influx and triggers release and depletion of the functionally separate readily releasable pool (RRP). The averaged total change in ΔC_m (IRP + RRP) of successfully electroporated and not electroporated cells was not significantly different (Fig. 5a), as were the sizes of the RRP. However, the IRP size was approximately twice as large in transfected cells, suggesting the step depolarizations used to drive IRP release may evoke modest release from the RRP in the transfected condition.

Chromaffin cells function as modified post-ganglionic sympathetic neurons, generating action potentials in response to splanchnic innervation. Hence, we evaluated whether electroporated cells retain the ability to generate action potentials with properties similar to freshly isolated chromaffin cells. Application of progressively stronger depolarizing current injections led to induction of action potential activity in both cell preparations. The V_m at which action potential(s) first appeared was not significantly

different (Fig. 5b), with an average spike number of 3.8 ± 1.4 for freshly dissociated cells and 4.2 ± 0.6 for transfected cells.

We also determined if there were kinetic differences in individual secretory granule fusion events. For this analysis, single cell amperometry data were generated using 5 μ m carbon fiber electrodes placed against cells. As catecholamines are released during fusion, they oxidize at the charged fiber tip, and the oxidation current is recorded. The initial event in exocytotic release is the formation of a fusion pore, a membrane-lined channel transiently uniting the vesicle lumen and extracellular space. In instances where the fusion pore does not expand rapidly, the catecholamine is slowly released, and a prespike foot (PSF) is seen, whose stability is represented by the duration, t_{foot} . When the vesicle collapses into the plasma membrane, releasing a large bolus of catecholamine, a large transient current is observed whose timing is represented by the duration of the half-height width, $t_{1/2}$. When cells were induced to secrete by application of a membrane depolarizing concentration of potassium (75 mM K^+ , 2 min), no differences were seen in t_{foot} or $t_{1/2}$ between transfected and non-transfected chromaffin cells (Fig. 5c), indicating electroporation does not compromise kinetic parameters of single fusion events.

Single cell assessment, whether utilizing capacitance or amperometric recordings, is valuable for

Fig. 5 Effect of electroporation protocol on measures of chromaffin cell physiology. **a** Comparison of membrane depolarization evoked secretory responses between non-transfected and transfected chromaffin cells as monitored by ΔC_m . Cells were given six 10 ms pulses to 0 mV from a holding potential of -80 mV to drive fusion of vesicles in the IRP, and six additional 100 ms pulses to fuse and deplete the RRP. Traces shown are averaged and baseline-subtracted. **b** Comparison of action potential (AP) threshold and frequency in transfected and freshly dissociated cells. Histogram (left) is averaged AP

threshold. Individual traces (right) are examples of the first appearance of APs. **c** Comparison of vesicle fusion events in EGFP-transfected and freshly dissociated cells using carbon fiber amperometry. An example current (left) shows a prespike foot (PSF). Histograms (right) quantify spike PSF, t_{foot} , and half-height width, $t_{1/2}$. **d** Secretion of hGH from population of electroporated and transfected chromaffin cells. Histogram quantifies hGH secretion in response to 5.6 mM K^+ (Basal) and 56 mM K^+ (Stim). Results are ave. $\pm$ SEM, $N = 2$

evaluating kinetic features of vesicle fusion and content release, respectively, with sub-millisecond resolution. By comparison, high-throughput evaluation of various conditions on secretion often uses an enzyme-linked immunosorbent assay (ELISA) method that monitors released secretory product after stimulation. One advantage of a high transfection efficiency method on mouse chromaffin cells is the ability to implement and use ELISA assays by transfection and overexpression of suitable regulated secretory products even when small number of cells

are available. Transfecting chromaffin cells with an hGH plasmid results in hGH, a 20 kD protein, being rapidly synthesized and packaged in dense-core vesicles such that its stimulated secretion can easily be detected with ELISA at 2 days post-transfection. In a co-transfection assay, hGH release may be measured from cells co-transfected with an hGH plasmid and a plasmid of interest. Moreover, since chromaffin cells do not endogenously express hGH, detection of hGH in the medium is a confirmation of both hGH protein expression and secretion (Wick et al. 1993).

Cotransfection in this manner allows one to rigorously probe the effects on secretion of co-expressed proteins or the knockdown of proteins through cotransfection of shRNA. Confirmation of successful application of the hGH assay is shown in Fig. 5d where a hGH secretion response was detected to 56 mM K⁺ stimulation in small populations of cultured mouse chromaffin cells.

For research to take full advantage of the small number of chromaffin cells harvested from genetically homogenous inbred mouse models, a new method of transfection was required. Here we present a method for chromaffin cells that does not involve the time, expense, and morphological and functional perturbations inherent in viral approaches. In addition, our approach results in an orders of magnitude greater transfection efficiency on primary chromaffin cells than when using the calcium phosphate method. Our optimization of a small-volume electroporation method achieves a transfection efficiency of 45 % with commercially available equipment. Further, we describe a novel method of chromaffin cell isolation and dissociation that results in a cell preparation that is ~92 % adrenal chromaffin cells. We investigated possible morphological and functional changes resulting from electroporation using immunocytochemistry, electrophysiology, and calcium imaging, and concluded the consequences were minimal. Finally, we demonstrate a cotransfection efficiency of over 95 %, making our procedure especially useful for high-throughput screening with a commercially available hGH ELISA.

Acknowledgments This work was supported by a grant from the NIDDK (NS053978) and is subject to the NIH Public Access Policy, and by University of Michigan Medical School Endowment for Basic Sciences.

Conflict of interest The authors have no conflicts of interest.

References

- Alesci S, Perera SM, Lai EW, Kukura C, Abu-Asab M, Tsokos M, Morris JC, Pacak K (2007) Adenoviral gene transfer in bovine adrenomedullary and murine pheochromocytoma cells: potential clinical and therapeutic relevance. *Endocrinology* 148:3900–3907. doi:[10.1210/en.2007-0521](https://doi.org/10.1210/en.2007-0521)
- Anantharam A, Bittner MA, Aikman RL, Stuenkel EL, Schmid SL, Axelrod D, Holz RW (2011) A new role for the dynamin GTPase in the regulation of fusion pore expansion. *Mol Biol Cell* 22:1907–1918. doi:[10.1091/mbc.E11-02-0101](https://doi.org/10.1091/mbc.E11-02-0101)
- Ashery U, Betz A, Xu T, Brose N, Rettig J (1999) An efficient method for infection of adrenal chromaffin cells using the Semliki Forest virus gene expression system. *Eur J Cell Biol* 78:525–532
- Aunis D, Hesketh JE, Devilliers G (1980) Immunohistochemical and immunocytochemical localization of myosin, chromogranin A and dopamine- β -hydroxylase in nerve cells in culture and in adrenal glands. *J Neurocytol* 9:255–274
- Axelrod D (1981) Cell-substrate contacts illuminated by total internal reflection fluorescence. *J Cell Biol* 89:141–145
- Barclay JW, Craig TJ, Fisher RJ, Ciuffo LF, Evans GJ, Morgan A, Burgoyne RD (2003) Phosphorylation of Munc18 by protein kinase C regulates the kinetics of exocytosis. *J Biol Chem* 278:10538–10545. doi:[10.1074/jbc.M211114200](https://doi.org/10.1074/jbc.M211114200)
- Duncan RR, Don-Wauchope AC, Tapechum S, Shipston MJ, Chow RH, Estibeiro P (1999) High-efficiency Semliki Forest virus-mediated transduction in bovine adrenal chromaffin cells. *Biochem J* 342:497–501. doi:[10.1042/0264-6021.3420497](https://doi.org/10.1042/0264-6021.3420497)
- Duncan RR, Greaves J, Tapechum S, Apps DK, Shipston MJ, Chow RH (2002) Efficacy of Semliki Forest virus transduction of bovine adrenal chromaffin cells—an analysis of heterologous protein targeting and distribution. *Ann NY Acad Sci* 971:641–646
- Ehrengruber MU, Schlesinger S, Lundstrom K (2011) Alphaviruses: Semliki forest virus and Sindbis virus vectors for gene transfer into neurons. *Curr Protoc Neurosci Chapter 4:Unit 4 22*
- Graham FL, van der Eb AJ (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52:456–467
- Graham ME, Fisher RJ, Burgoyne RD (2000) Measurement of exocytosis by amperometry in adrenal chromaffin cells: effects of clostridial neurotoxins and activation of protein kinase C on fusion pore kinetics. *Biochimie* 82:469–479. doi:[http://dx.doi.org/10.1016/S0300-9084\(00\)00196-6](https://doi.org/10.1016/S0300-9084(00)00196-6)
- Jordan M, Wurm F (2004) Transfection of adherent and suspended cells by calcium phosphate. *Methods* 33:136–143. doi:[10.1016/j.ymeth.2003.11.011](https://doi.org/10.1016/j.ymeth.2003.11.011)
- Kee ST, Gehl J, Lee EW (2011) Clinical aspects of electroporation. Springer, New York
- Kotnik T, Miklavcic D (2000) Analytical description of transmembrane voltage induced by electric fields on spheroidal cells. *Biophys J* 79:670–679. doi:[10.1016/S0006-3495\(00\)76325-9](https://doi.org/10.1016/S0006-3495(00)76325-9)
- Li XY, Drakulich DA, Zhang PJ, Shen ML, Weber GA, Ikezu T, Hexum TD (2002) Transduction of bovine adrenal chromaffin cells using a recombinant adenovirus expressing GFP. *J Neurosci Methods* 122:91–96. doi:[10.1016/s0165-0270\(02\)00295-9](https://doi.org/10.1016/s0165-0270(02)00295-9)
- Rols MP, Teissie J (1990) Electroporation of mammalian cells. Quantitative analysis of the phenomenon. *Biophys J* 58:1089–1098. doi:[10.1016/S0006-3495\(90\)82451-6](https://doi.org/10.1016/S0006-3495(90)82451-6)
- Teissie J, Rols MP (1993) An experimental evaluation of the critical potential difference inducing cell membrane electroporation. *Biophys J* 65:409–413. doi:[10.1016/S0006-3495\(93\)81052-X](https://doi.org/10.1016/S0006-3495(93)81052-X)
- Voets T, Neher E, Moser T (1999) Mechanisms underlying phasic and sustained secretion in chromaffin cells from mouse adrenal slices. *Neuron* 23:607–615

Wick PF, Senter RA, Parsels LA, Uhler MD, Holz RW (1993) Transient transfection studies of secretion in bovine chromaffin cells and PC12 cells. Generation of kainate-sensitive chromaffin cells. *J Biol Chem* 268:10983–10989

Wilson SP, Smith LA (1997) Addition of glycerol during DNA exposure enhances calcium phosphate transfection. *Anal Biochem* 246:148–150. doi:[10.1006/abio.1997.2005](https://doi.org/10.1006/abio.1997.2005)