Amperometric detection of stimulus-induced quantal release of catecholamines from cultured superior cervical ganglion neurons

(amerometry/sympathetic neurons/synapse formation/stimulus secretion coupling/black widow spider venom)

ZHUAH ZHOU* AND STANLEY MISLER

Departments of Medicine and Cell Biology/Physiology, Washington University School of Medicine, St. Louis, MO 63110

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ABSTRACT Amperometry has been used for real-time electrochemical detection of the quantal release of catecholamines and indolamines from secretory granules in chromaffin and mast cells. Using improved-sensitivity carbon fiber electrodes, we now report the detection of quantal catecholamine release at the surface of somas of neonatal superior cervical ganglion neurons that are studded with axon varicosities containing synaptic vesicles. Local application of a bath solution containing high K⁺ or black widow spider venom, each of which greatly enhances spontaneous quantal release of transmitter at synapses, evoked barrages of small-amplitude (2–20 pA), short-duration (0.5–2 ms) amperometric quantal “spikes.” The median spike charge was calculated as 11.3 fC. This figure corresponds to 3.5 × 10⁴ catecholamine molecules per quantum of release, or ~1% that evoked by the discharge of the contents of a chromaffin granule.

Amperometry with microelectrodes has been a very useful technique for monitoring exocytotic release of catecholamines and indolamines from adrenal chromaffin cells and mast cells that contain these messenger molecules in large, dense-core granules (1). By positioning a carbon fiber electrode, held at a fixed potential, near the surface of these cells, it is possible to record, in real time, over discrete regions of the cell, single spike-like events thought to represent the synchronized release of the transmitter content of such a granule (2). In contrast, nerve terminals contain smaller-diameter synaptic vesicles and make close contact with chemoreceptive "follower" cells. In principle, these features make amperometry less appealing than postsynaptic electrophysiological recording as an instantaneous assay for release. However, there are several situations where direct chemical detection of the secreted transmitter is preferable or necessary: (i) at developing cell-to-cell contacts where maturation of presynaptic release sites may precede that of the postsynaptic receptors; (ii) at synapses where the identity of the chemical transmitter or quantitation of the transmitter content of a granule is sought; and (iii) at synapses where the transmitter activates a slow cascade of postsynaptic second-messenger-related events, thus obscuring the kinetics of release.

Recently we have begun to use amperometry to investigate quantal release of catecholamines from developing superior cervical ganglion (SCG) neurons maintained in culture under conditions where they release catecholamines on stimulation with high-K⁺ solutions (3). Axons growing from these cells form varicosities, containing 30–90-nm-diameter synaptic vesicles, which contact cell bodies or other axons (4); however, evidence for functional synaptic transmission is lacking (5). By positioning a carbon fiber electrode into the cleft between somas of these SCG neurons, we have recorded "amperometric spikes" in response to the application of either high-K⁺ Ringer's solution (6) or crude black widow spider venom (BWSV) (7), two nerve terminal secretagogues. This response occurs only when the electrode was held at a voltage promoting catecholamine oxidation. The charge transfer per quantal "spike" is ~1% that evoked by release of the contents of a chromaffin granule. These results suggest that amperometry may be useful in monitoring stimulated quantal release of transmitter from developing nerve terminals before true synapse formation.

MATERIALS AND METHODS

Cell Preparation and Treatment. SCG were removed from neonatal rats and dissociated using described methods (8). Single chromaffin cells were prepared from rat adrenal medulla as described (9). Cells were plated on collagen-coated glass coverslips and maintained in primary culture at 37°C in Eagle's minimal essential medium (GIBCO)/10% fetal bovine serum (HyClone)/penicillin at 10 units/ml/streptomycin at 100 μg/ml in 95% air/5% CO₂. Nerve growth factor (50 ng/ml; 2.5 S) was added to the medium used for SCG neurons. Experiments were done at room temperature (20–22°C) on cells cultured for 2–10 days. The standard bath or extracellular solution (ES) contained 135 mM NaCl, 5.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 20 mM Hepes titrated to pH 7.4 with NaOH.

Amperometric Measurements to Determine Catecholamine Release. Amperometric measurements were made with polyethylene-insulated carbon fiber electrodes fabricated according to Chow et al. (10) with subsequent modification to optimize high-resolution recording (11). The tip of a freshly prepared or recut polyethylene-insulated carbon fiber electrode, held at a potential of +780 mV by an EPC-7 patch-clamp amplifier, was "micro"-positioned to just touch the surface of SCG neurons at the clefts between somas in a small cell aggregate. To evoke amperometric signals, test solutions were applied by a puffert pipette, a low-resistance patch pipette positioned within 50 μm of the cell. Two types of test solutions were used: (i) high-K⁺ (62.5 mM) ES with or without added Ca²⁺ (i.e., a modified ES in which 57 mM KCl was isosmotically substituted for NaCl in the presence or absence of 6 mM CaCl₂), and (ii) a dilute solution of BWSV in ES in [i.e., an extract of two venom apparatus of a Latrodectus magnus (7) prepared in 100 μl of 166 mM NaCl and then diluted 1:9 with ES].

Electrophysiology and Data Acquisition. Perforated-patch electrophysiological recordings were made on somas of SCG neurons using published techniques (11). The tip of the patch pipette was filled with an internal solution (IS) containing 63.7 mM KCl, 28.35 mM K₂SO₄, 47.2 mM sucrose, 11.8 mM NaCl, 1 mM MgCl₂, and 20 mM Hepes titrated to pH 7.3 with KOH. The pipette was backfilled with a modified IS which also

Abbreviations: SCG, superior cervical ganglion; ES, extracellular solution; IS, internal solution.

*To whom reprint requests should be addressed: Renal Division (Yalem 815), The Jewish Hospital of St. Louis, 216 South Kingshighway Boulevard, St. Louis, MO 63110.
Neurobiology: Zhou and Misler

RESULTS

Fig. 1 demonstrates that SCG neurons maintained in culture for 3–10 days often are arrayed as somal aggregates attached by connectives consisting of axon bundles in fascicles (e.g., Fig. 14). Electron microscopic studies of cultures maintained under conditions similar to ours demonstrated that ~1-μm-diameter axon varicosities, containing synaptic vesicles, occasionally contact cell somas (refs. 4 and R. Schmidt, personal communication). Images of aggregates of SCG neurons immunostained for the synaptic vesicle antigen SV-2, obtained by serial confocal fluorescence microscopy, show that these vesicle-containing axon varicosities occur with greater frequency in 2-μm-thick optical sections nearest the exposed surface of these aggregates (Fig. 1C) than in sections immediately below, where the outlines of somas and cell nuclei are clearly evident (Fig. 1D). These results, typically seen in somal aggregates in 3- to 8-day-old cultures, suggest that axons approach the somatic aggregate from its periphery as bundles, ramify, and then encircle the somas near their exposed surface. On the basis of this, we predicted it might be possible to amperometrically monitor quantal catecholamine release from such aggregates during focal stimulation by a chemical secretagogue, by positioning a high-sensitivity carbon fiber electrode onto the clefts between somas (see Fig. 1B). Fig. 2 presents electrochemical data supporting this last prediction. Fig. 2A shows that with the tip of a 7-μm-diameter carbon fiber electrode positioned into the cleft between cell bodies of an aggregate of SCG neurons, local application of high-K+ ES (containing 2 mM CaCl2) via a puffer pipette results in a barrage of amperometric spikes that commences within 3–5 s of the onset of the puff and then ends within 10–15 s of its termination. These spike events, ranging from <1 to 20 pA in amplitude, are seen when the electrode is held at +780 mV (traces 1 and 3), a potential sufficient to oxidize catecholamines but are not seen at +100 mV (trace 2), a potential at which catecholamines are not readily oxidized. Repeated puffs of the high-K+ ES, applied at 1- to 2-min intervals, often progressively reduce the number of spikes per discharge and,

contained nystatin (250 μg/ml). Electrical recordings were made once the access resistance from the pipette to the cell interior fell to <35 MΩ, usually 1–5 min after achieving a pipette-to-membrane seal resistance of >2–3 GΩ. Action potentials were evoked and recorded with an EPC-9 amplifier (HEKA Electronic, Lambrecht, F.R.G.) controlled by an Atari computer. Data from the patch-clamp amplifiers were acquired onto a Macintosh Quadra 650 computer running PULSE CONTROL software (12) using an ITC-16 analog/digital converter interface (Instrutech, Mineola, NY). Amperometric data were low-band-pass filtered at 0.3–0.5 kHz, except where otherwise indicated; membrane potential data was gathered at 2.5 kHz. All data were sampled at 3.3 kHz. Amperometric spikes were analyzed, and histograms of their features were compiled by using macro programs written by Robert Chow, (Max Planck Institute for Experimental Medicine, Göttingen, Germany), and one of us (Z.Z.) with IGOR software (WaveMetrics, Lake Oswego, OR).

Immunohistochemistry. Cells plated on glass coverslips were fixed for 10 min in 4% (vol/vol) paraformaldehyde at 37°C, permeabilized by incubation with 0.1% Triton X-100/Ringer’s solution for 2 min at room temperature, and then exposed for 2 hr at room temperature to a 1:300 dilution of mouse anti-SV-2 antibody (J. Huettner, Washington University, St. Louis) in ES/3% bovine serum albumin. SV-2 is a ubiquitous synaptic vesicle antigen, which may represent a subunit of the carrier mechanism that concentrates transmitter in the vesicle lumen (13). Cells were then incubated for 30 min with a fluorescein isothiocyanate-tagged goat anti-mouse antibody diluted 1:200 in bovine serum albumin/ES. Rinsed coverslips were mounted on a microscope slide and viewed with a Bio-Rad model MRC-1000 laser scanning confocal microscope assembled on a Zeiss Axoplan body. The krypton-argon mixed-gas laser scanned the microscope field, maximally exciting fluorescein isothiocyanate at 488 nm. Fluorescent light emission was viewed through appropriate filters. Digitized images of horizontal slices were acquired at 1- to 2-μm steps.

Fig. 1. Anatomic features of cultured SCG neurons suggesting the feasibility of amperometric recording. (A) Phase-contrast photomicrograph of a typical region of a living culture of SCG neurons showing aggregates of neuronal somas attached by axons often in fascicles. (B) Diagram of electrode and pipette configurations for stimulation/recording. A polyethylene-coated carbon fiber electrode (CFE) is gently wedged into a cleft between two somas. The puffer pipettes are positioned for delivery of chemical stimulus ~20–50 μm from the somal aggregate. The patch-pipette electrode is sealed to the soma. (C and D) Serial confocal micrographs of a fixed, permeabilized culture immunostained for synaptic vesicle antigen SV2. Note the increased abundance of strands of punctate fluorescence in the topmost 2-μm section of the somal aggregate (C) as compared with the 2-μm section just below it (D). [Bar = 100 μm (A) and 25 μm (C).]
in the case of Fig. 2B, result in total failure by the fifth puff. Recovery was not seen after several minutes of rest, suggesting depletion of releasable quantal stores. Similar results were seen with >20 aggregates cultured for 2–10 days. Over this interval, the probability of recording a barrage of spikes from an aggregate increased with time in culture.

Fig. 3 presents evidence that the spike barrages evoked by puffs of high-K⁺ ES, like K⁺-induced quantal release from classical synapses, depend on adequate extracellular [Ca²⁺] and membrane depolarization. When an aggregate of SCG neurons is bathed in modified ES/0.1 mM Ca²⁺, puffs of high-K⁺ ES containing <20 μM Ca²⁺ evoke few amperometric spikes, whereas alternating puffs of high-K⁺ ES/6 mM Ca²⁺ evoke many more (Fig. 3A). When amperometric recording is combined with patch-clamp recording from the soma of an adjacent cell, puffs of high-K⁺ ES, which provoke barrages of amperometric events, also produce sufficient depolarization in the SCG soma to sustain massive membrane potential, often to a plateau value of ~15 mV (Fig. 3B). This behavior is consistent with the hypothesis that application of high-K⁺ opens high-voltage-activated, perhaps N-type, Ca²⁺ currents, like those recorded from the somas of these cells (14).

BSWV and its active principle, cr-latrophin, enhance exocytotic release and depletion of the vesicular store of neurotransmitter from a host of nerve terminals, including nerve terminals in the iris of the eye, an in vivo target of adult SCG neurons (7). These substances also produce massive catecholamine release from adrenal chromaffin cells (15). The venin and its toxic action, in part, by inserting nonselective cation channels in the plasma membrane (16, 17). Fig. 4 demonstrates that, with both cell types, application by puffer pipette of a dilute extract of BWSV results in a barrage of amperometric events with similar amplitude as those evoked by high-K⁺ ES. In contrast to those evoked by puffs of high-K⁺ ES, the spike barrages evoked by the equally brief puffs of the venom are delayed in onset by up to 10–20 s and persist for tens of seconds after conclusion of the puff (n = 4 paired experiments).

The venom-induced discharges provided sufficient quantities of events for comparison of the characteristics of amperometrically detectable quanta in chromaffin cells vs. catecholaminergic neurons in culture (Fig. 5). Three features were tabulated: (i) half-height duration (t₁/₂), (ii) amplitude, and (iii) total charge (Q) (i.e., the integral of the spike current over time). An underlying assumption of amperometry is that when a carbon fiber electrode with a large surface area is positioned within several microns of localized sites of release of an oxidizable transmitter, the electrode will "capture" and oxidize most or all of the molecules released (2). Having

Fig. 3. "Physiological" features of high-K⁺-induced quantal release monitored by amperometry. (A) Dependence on extracellular Ca²⁺. When puffer pipettes containing high-K⁺ ES with either 0.2 or 2 mM Ca²⁺ were positioned equidistantly from the region of somal aggregate being recorded, the puff of high-K⁺ ES/6 mM Ca²⁺ is repeatedly much more effective in evoking spikes. (B) Comparison of time courses of amperometric spike barrages and cell depolarization. Simultaneous amperometric and patch-clamp recording from an adjacent soma demonstrates that KCl-induced barrages of spikes coincide with the development of a plateau-phase depolarization in the soma, sometimes preceded by a train of action potentials (see inset). In this experiment, the amperometric current (Iamp) was low-band-pass filtered at 30 Hz. Vm, membrane potential.
determined $Q$, the total number of catecholamine (CA) molecules released as a packet or quantum is calculated as:

$$Q = \frac{(1 \text{ CA molecule}/2e^-) \times (1e^-/1.6 \times 10^{-19} \text{ coulomb})}{2e^-}$$

assuming two electro-oxidations per catecholamine molecule and the absence of other transiently released, oxidizable molecular species. To date, there is no evidence that other soluble contents of catecholamine-containing vesicles—e.g., ATP or chromogranin-like peptides—undergo redox reactions at $+780 \text{ mV}$. Note that, despite their small sizes, spike events recorded from SCG neurons have very rapid rise times and short durations, suggesting that release is occurring very near the electrode. In SCG neurons, the median spike charge is 11.3 fC (vs. a mean of 13.5 fC), which corresponds to the release of $3.5 \times 10^6$ catecholamine molecules. In chromaffin cells, the median spike charge is 890 fC (vs. a mean of 1622 fC), which corresponds to the release of $2.9 \times 10^6$ catecholamine molecules from a chromaffin granule.

**DISCUSSION**

Electrochemical detection of catecholamines and indolamines has provided a relatively noninvasive, self-contained in vitro tool for detection of exocytotic release of these transmitter substances from adrenal chromaffin cells and mast cells with speed, sensitivity, and specificity approaching that of postsynaptic electrophysiological measurements. Here we have applied improved sensitivity carbon fiber amperometry to detect quantal release of neurotransmitter from neurons. Under culture conditions where (i) catecholamines are secreted in the bath and (ii) vesicle-rich axon varicosities contact somas, local applications of either of two known nerve-terminal secretagogues, elevated extracellular $K^+$ or BWSV, evoke discrete, spike-like, small-amplitude amperometric events from the surface of somas of cultured SCG neurons, provided the electrode is held at a potential favoring catecholamine oxidation. The rapid rise times and brief half-height durations of these spikes suggest that they are registering nearby secretion of multimolecular packets of quanta of transmitter. Assuming the total oxidizable content of the packet consists of catecholamines and is rapidly and completely oxidized at the electrode surface, from the median spike charge, we estimate the packet contains $\sim30,000$ catecholamine molecules.

To be sure, quantal release of transmitter has been detected from somas and along lengths of neurites, as well as at neural growth cones in growing neurons—e.g., acetylcholine release from *Xenopus* spinal cord (18). However, several lines of
evidence suggest that nerve terminals are the source of our amperometrically detected release. (i) Over the first 5 days of culture, the increased perisomal density of immunohistochemically identified varicosities (up to 10 per soma) roughly parallels our increased success, over time, at detecting release events amperometrically. (ii) Available electron micrographs of cultures maintained under conditions similar to ours reveal that individual axon varicosities that contact somas contain up to hundreds of vesicles. However, these micrographs fail to show collections of synaptic vesicles within the soma or dendrites. In addition, we favor the idea that quanta release is occurring in the absence of functional synaptic transmission.

Although SCG neurons maintained in culture for >20 days develop cholinergic chemical contacts among themselves, currently there is no evidence for chemical transmission in more short-term cultures where a catecholaminergic phenotype is maintained (5). Furthermore, in the six experiments (e.g., Fig. 3B), no transient changes in somal membrane potential were seen simultaneously with amperometric spikes.

Our estimate of the number of catecholamine molecules per quantum of release from SCG neurons seems plausible when we compare, between SCG neurons and adrenal chromaffin cells, the relative size of catecholamine-containing granules and the relative amperometric spike charge attributable to quanta release. With chromaffin cells, strong arguments have been made that the release of the contents of a single 250- to 300-nm-diameter spherical granule results in an amperometric spike (2). Here we calculate its total charge to be 890 fC. From recordings made with SCG neurons, under identical conditions, we calculate a spike charge of 11.3 fC, implying that the quantum of release from the neuron is nearly 80-fold smaller than for chromaffin cells. Assuming similar catecholamine concentrations in granules from both types of cells, the quantity of catecholamine molecules contributing to the spike recorded from the neurons could be accommodated by a spherical vesicle with a diameter of 60–70 nm. This range is squarely in the range of measured vesicle diameters (30–90 nm). By a similar argument, our results are also consistent with recent amperometric data from carotid body glomus cells and primary chemoreceptor cells, which contain 100-nm-diameter dense-core vesicles and have 5-fold larger spike charges than in our neurons (19). Biochemical estimates of vesicular catecholamine content of synaptic vesicles range from 15,000 molecules per 100-nm-diameter dense-core vesicle to several thousand per 40-nm-diameter clear vesicle (20); these may be underestimated due to catecholamine leakage during vesicle isolation. Under our culture conditions (i) both sizes of catecholamine-containing vesicles are present, and they may be variably filled with catecholamine and/or acetylcholine (21), and (ii) our spike-charge histograms are not clearly unimodal. These features preclude clear association of spike charge to vesicle size. However, our estimate of the molecular contents of a quantum is also in the range of an available estimate of the number of molecules needed to produce a quantum of transmitter release measured electrophysiologically. Close iontophoresis, onto a muscle end-plate, of 7,000–10,000 molecules of acetylcholine, is needed to mimic a miniature synaptic potential (22).

Our results suggest that amperometry, a physical technique, complements two previously described biosensor techniques of comparable sensitivity, specificity, and speed, for use in the rapid, specific, and sensitive detection of transmitter release from terminals in the absence of stable synaptic connections. These biosensor techniques are (i) the use of excised outside-out patches of myotube plasma membrane, rich in nicotinic receptor channels, used to “sniff” acetylcholine released by growth cones of spinal neurons, and (ii) the temporary juxtaposition of whole myotubes against these growth cones to form a temporary synapse-like connection (18, 23, 24). Both bio-

sensor techniques require receptor-activated ion channels that are not present in a catecholamine-sensing system. The detection capacity of the “sniffer” pipette is limited by the number of functional nicotinic receptor channels encompassed in a membrane patch and the tendency for the gating of these channels to degrade with time after patch excision. The cell-juxtaposition approach requires complex coculture of at least two cell types. In contrast, the carbon fiber electrode can be consistently fabricated without resort to biological material, does not appear to desensitize over minutes, and has a much larger sensor area (25–49 μm²), thereby permitting its use in quantitating the content of oxidizable transmitter in a secretory granule. Attempts to expand the applicability of the carbon fiber electrode beyond catecholamines and indolamines to reactable peptides have been reported (25).

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