Distinguishing splanchnic nerve and chromaffin cell stimulation in mouse adrenal slices with fast-scan cyclic voltammetry

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Electrical stimulation is an indispensable tool in studying electrically excitable tissues in neurobiology and neuroendocrinology. In this work, the consequences of high-intensity electrical stimulation on the release of catecholamines from adrenal gland slices were examined with fast-scan cyclic voltammetry at carbon fiber microelectrodes. A biphasic signal, consisting of a fast and slow phase, was observed when electrical stimulations typically used in tissue slices (10 Hz, 350 μA biphasic, 2.0 ms/phase pulse width) were applied to bipolar tungsten-stimulating electrodes. This signal was found to be stimulation dependent, and the slow phase of the signal was abolished when smaller (≤250 μA) and shorter (1 ms/phase) stimulations were used. The slow phase of the biphasic signal was found to be tetrodotoxin and hexamethonium independent, while the fast phase was greatly reduced using these pharmacological agents. Two different types of calcium responses were observed, where the fast phase was abolished by perfusion with a low-calcium buffer while both the fast and slow phases could be modulated when Ca2+ was completely excluded from the solution using EGTA. Perfusion with nifedipine resulted in the reduction of the slow catecholamine release to 29% of the original signal, while the fast phase was only decreased to 74% of predrug values. From these results, it was determined that high-intensity stimulations of the adrenal medulla result in depolarizing not only the splanchnic nerves, but also the chromaffin cells themselves resulting in a biphasic catecholamine release.

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KCl, 1.25 NaH2PO4, 3 MgCl2, 10 glucose, and 10 HEPEs, and its pH was adjusted to 7.4. Excess fat was removed from the glands, and the glands were embedded in a 3% agarose (wt/vol) in low-Ca2+ BBS (Promega). Once cooled, agarose blocks containing the glands were secured onto a Teflon specimen holder that was used to orient the largest base of the gland parallel to the slicing blade. The glands were cut into slices of ~200 μm in thickness using a vibratome slicer filled with low-Ca2+ BBS (Campden Instruments, World Precision Instruments, Leics, UK).

Slices were immediately placed into a perfusion chamber (open diamond bath-heated chamber, RC-22, Warner Instruments, Hamden, CT) and secured with a stainless steel Lyca thread anchor (1.5 mm-2.0 mm thread spacing, Warner Instruments). The chamber was continually perfused with 95% O2-5% CO2 saturated BBS, at a rate of 1–2 ml/min with the same composition as the low-Ca2+ BBS, except with 2 mM CaCl2 and 1 mM MgCl2 at pH 7.4. To ensure physiological conditions and to minimize slice perturbations, the temperature of the perfusion chamber was kept at 37°C using an automatic temperature controller (Warner Instruments). All of the experiments were completed within a 10-h post mortem period.

Fast-scan cyclic voltammetry. Carbon fiber elliptical microelectrodes were fabricated as previously described (9, 20). Before use, carbon fiber microelectrodes were soaked in an activated carbon/2-

Characteristics of evoked catecholamine release. In prior work we showed that local electrical stimulation of adrenal medullary slices with stimulus trains consisting of 10 biphasic pulses, 1 ms/phase, 150 μA at 10 Hz results in catecholamine release that increases during the stimulation and then decreases 1–2 s after the stimulation is terminated (37). Furthermore, we showed that the pharmacological manipulation during such stimulations was consistent with activation of the splanchic nerve causing release of acetylcholine. We refer to this as the fast phase of catecholamine release. However, quite different results were obtained with an identical stimulation except with a greater amplitude (n = 3, Fig. 1A). With stimulus amplitude of 450 μA, catecholamine release is still evoked, but it lasts over a longer time frame. A maximum corresponding to the fast phase is observed shortly (1–2 s) after the stimulation is terminated, and it is followed by a broad, larger increase that maximizes ~8–10 s after the stimulation. We refer to this second feature as the slow phase of release. As the stimulus amplitude was diminished, the slow phase diminished more rapidly than the fast phase. Similar behavior was seen within chromaffin-cell clusters throughout the adrenal medulla. In some instances, the slow phase was larger in amplitude than the fast phase and overwhelmed it. The cyclic voltammograms (Fig. 1, B and C, encoded as color plots) show that catecholamine release is the primary contributor to both phases. The maximum amplitude of each phase as a function of stimulus intensity is shown in Fig. 1D.

To probe further the effects of electrical stimulation parameters on catecholamine release, various stimulation pulse widths were applied to the slice using a 350-μA stimulation amplitude. Stimulating pulse widths (per phase) of 2.0 ms, 1.5 ms, 1.0 ms, and 0.5 ms were employed (pulse widths were delivered in random order). The slow phase was not present with stimulation widths of ≤1 ms per phase (n = 5, Fig. 2). The remaining experiments described here were designed to probe the origin of the slow release phase and used relatively large stimulations (350 μA and 2.0 ms/phase width).

Modulation of catecholamine release by ions. We investigated the role of cholinergic receptors on the slow phase of catecholamine release. As previously shown, the fast phase at +600 mV (the peak current for catecholamine oxidation) was extracted from the color plots. Since the electrochemical signals recorded in the adrenal gland are a mixture of epinephrine, and norepinephrine (37), electrodes were post calibrated in a flow injection system with 5 μM epinephrine, norepinephrine, and a 50:50 mixture of 2.5 μM of each catecholamine. Concentrations measured during application of pharmacological agents are reported as values normalized to currents recorded before drug application.

Statistical analysis. Statistical analysis was done in GraphPad Prism (GraphPad Software, San Diego, CA). Student’s t-test (unpaired) was used to test for significant differences between data sets. Data were considered significantly different at the 95% confidence level; n is number of slices, unless noted otherwise.

RESULTS

Pharmacology. Pharmacological agents employed herein include thapsigargin (THG), TTX, Hex, 4-diphenylacetoxy-N-methylpiperidine (4-DAMP), ethylene glycol tetraacetic acid (EGTA), atropine, and nifedipine. All were from Sigma-Aldrich (St. Louis, MO) and were used as received. Drugs were dissolved in low-Ca2+ or recording BBS and superfused through a slice chamber at a rate of ~2 ml/min.

To probe further the effects of electrical stimulation parameters on catecholamine release, various stimulation pulse widths were applied to the slice using a 350-μA stimulation amplitude. Stimulating pulse widths (per phase) of 2.0 ms, 1.5 ms, 1.0 ms, and 0.5 ms were employed (pulse widths were delivered in random order). The slow phase was not present with stimulation widths of ≤1 ms per phase (n = 5, Fig. 2). The remaining experiments described here were designed to probe the origin of the slow release phase and used relatively large stimulations (350 μA and 2.0 ms/phase width).

Modulation of catecholamine release by ions. We investigated the role of cholinergic receptors on the slow phase of catecholamine release. As previously shown, the fast phase
was diminished in the presence of 100 μM Hex, a nicotinic acetylcholine receptor blocker. In contrast, the slow phase was only marginally affected by Hex (representative traces are shown in Fig. 3A and average maximal amplitudes are shown in Fig. 3B; n = 5). Previous work has shown that splanchnic nerve-dependent catecholamine release measured by FSCV is independent of muscarinic receptors (37). Perfusion with 1.0 μM 4-DAMP (n = 3, data not shown), a selective muscarinic receptor antagonist, also did not affect the slow phase of electrically stimulated release. Furthermore, perfusion with 0.5 μM atropine (n = 3, data not shown), a high-affinity muscarinic receptor antagonist, had no effect on the biphasic release. The inability of cholinergic antagonists to affect the slow phase of catecholamine release indicates that acetylcholine released from the splanchnic nerve is unnecessary for this type of release.

To evaluate the involvement of Na⁺-dependent action potentials in slow phase catecholamine release, 1 μM TTX, an inhibitor of voltage-gated Na⁺ channels (10), was perfused through the slice. The slow phase of the electrically stimulated release was found to be much less sensitive to TTX than the fast phase, which was abolished almost immediately upon perfusion as previously reported (representative example shown in Fig. 3C). A summary of the effects of TTX on the maximum amplitude of the two phases is shown in Fig. 3D (n = 5).

**Biphasic signal dependence on intra- and extracellular Ca²⁺ concentration.** To explore the Ca²⁺ concentration ([Ca²⁺]) dependence of the slow phase of electrically stimulated release, the slices were perfused with BBS containing 0.1 mM Ca²⁺. In this media, the fast phase release component was eliminated while the slow phase remained intact (representative example shown in Fig. 4A). Data from multiple trials are summarized in Fig. 4B (n = 3). When the slice was perfused with BBS containing 0 mM Ca²⁺ and 1 mM EGTA, both the fast and slow release was abolished within 15 min (representative example in Fig. 4C). Both phases partially recovered when extracellular Ca²⁺ levels were returned to their normal level (2 mM). These data are summarized in Fig. 4D (n = 5). Thus, while the slow phase does not require concentrations of physiological levels of extracellular Ca²⁺, it does require some Ca²⁺ for release to occur.

To test the possibility that the slow phase requires release of cytoplasmic Ca²⁺ levels from intracellular stores such as endoplasmic reticulum, the slices were perfused with THG. THG is a pharmacological agent that increases cytosolic Ca²⁺ levels by binding to sarco(endo)plasmic reticulum Ca²⁺-ATPase enzymes and preventing Ca²⁺ from being pumped into the sarcoplasmic and endoplasmic reticula (29). Perfusion of 1 μM THG dissolved in low-[Ca²⁺] BBS buffer for 1 h did not have
an effect on either the fast or the slow components of electrically stimulated release \((n = 4, \text{data not shown})\).

**Effect of L-type Ca\(^{2+}\) channel blocker on slow release.** It has been shown that L-type voltage-gated Ca\(^{2+}\) channels (VGCCs) are located on chromaffin cells but not on the splanchnic nerve and that they play a key role in the homeostatic mechanism of catecholamine release \((1, 25, 41)\). To evaluate the role of these channels in the different phases of release, we perfused 10 \(\mu M\) nifedipine, an L-type Ca\(^{2+}\) channel blocker, through the slice. The maximal amplitude of the slow phase was decreased to 29\% of its original value by this perfusion while 74\% of the fast phase remained. Figure 5A shows biphasic release before nifedipine is put on board, while Fig. 5B is the observed release 10 min after nifedipine application, and, finally, Fig. 5C shows that the slow phase was completely abolished by nifedipine 20 min after nifedipine had been put on board. A summary of the effects of nifedipine on the biphasic response is shown in Fig. 5D \((n = 5)\).

**Biphasic signal dependence on the distance between working and stimulating electrodes.** To probe the effect of the location of the stimulating electrode relative to the carbon fiber microelectrode, the stimulating electrode was moved away from the working electrode in 50-\(\mu M\) increments and release was evoked (Fig. 6). At increasing distances, both the fast and slow phases decrease in intensity. The slow phase is diminished to a greater degree, however, allowing the fast phase to predominate at greater separations of the stimulating electrode and the carbon fiber microelectrode. At the greatest distances between the electrodes, where only the fast phase was present \((200 \mu M)\), perfusion with 100 \(\mu M\) Hex (Fig. 7A) or 1 \(\mu M\) TTX (Fig. 7B) reversibly eliminated the stimulated release. These data are summarized in Fig. 7C \((n = 3, P < 0.001 \text{ for each drug})\).

**DISCUSSION**

Prior work in the perfused adrenal gland demonstrated that electrical stimulation could evoke catecholamine release by two mechanisms: indirectly by depolarization of the splanchnic nerve or directly by depolarization of the chromaffin cells themselves \((48, 50)\). Catecholamine release arising from splanchnic nerve stimulation occurs because released acetylcholine from the splanchnic nerve activates nicotinic receptors on chromaffin cells. The prior work demonstrated that direct depolarization of chromaffin cells required higher stimulation intensity than was required to activate the splanchnic nerve. The present work in which release is detected with a microsensor inserted directly into slices containing the adrenal medulla supports the prior findings and provides additional information concerning the temporal and spatial dependence of the two mechanisms that cause catecholamine release. The fast phase of release clearly originates from splanchnic nerve stimulation...
because it is blocked by a nicotinic antagonist. The slow phase of release is unaffected by nicotinic antagonists, but it is inhibited by an L-type calcium channel antagonist. This demonstrates that the slow phase of release originates from direct depolarization of chromaffin cells because acetylcholine release from the splanchnic nerve is known to be unaffected by L-type calcium channel antagonists, whereas their presence inhibits catecholamine release from electrically excited adrenal glands (1, 41).

Evidence that the splanchnic nerve is not involved in the slow phase of catecholamine release also comes from the experiments with TTX incubation. Consistent with our prior work, TTX blocked the splanchnic-nerve dependent component of release of catecholamines, demonstrating the need for action potential propagation supported by voltage-dependent Na⁺ channels (19, 51). Chromaffin cells are drastically different, however, because they do not require the opening of Na⁺ for catecholamine release. Rather, direct depolarization of the chromaffin cell can cause release due to opening of VGCCs (16, 43). This is an important distinction because it illustrates the different mechanisms in play during high-intensity electrical stimulation and the resulting biphasic release.

The splanchnic nerve consists of fine fibers closely associated with Schwann cells that innervate the chromaffin cells (12, 44). As such, they would be expected to be depolarized by milder electrical stimulations than the chromaffin cells themselves. The splanchnic nerve can be depolarized over large distances (over 200 μm) due to the morphology of its innervation throughout the gland while still being susceptible to pharmacological manipulations (12, 18, 44). This propagation of the stimulation through the splanchnic nerve network allows release to be evoked from chromaffin cells throughout the tissue including those directly adjacent to the microelectrode. Release adjacent to the microelectrode would appear rapidly, leading to the fast appearance of released catecholamines. While chromaffin cells themselves are electrically excitable (22), their lack of myelination and their larger size requires that they receive a greater stimulus intensity to cause exocytosis (28). Thus, the chromaffin cells most likely to be directly depolarized by the stimulation are those that are closest to the stimulating electrode. For detection, the released catecholamines from direct chromaffin cell stimulation must diffuse to the detecting microelectrode, a time-dependent process. Consistent with this interpretation, the amplitude of the slow phase diminished more rapidly than the fast phase when the stimulating electrode was moved further away from the carbon fiber microelectrode.

We also considered other mechanisms that could be the origin of the slow phase of release. One is the interplay of intracellular [Ca²⁺] and muscarinic cholinergic receptors. At
isolated chromaffin cells, muscarinic receptor activation has been shown to cause release of Ca\(^{2+}\) from intracellular stores (11, 39, 49). Exocytosis, when evoked by mobilization of intracellular Ca\(^{2+}\) stores, is temporally delayed in comparison to endogenous acetylcholine induced response (30). However, the slow phase did not display any dependence on THG, a pharmacological agent that causes release of Ca\(^{2+}\) from intracellular stores. Furthermore, the slow phase was insensitive to both atropine and 4-DAMP, a group of highly selective muscarinic receptor blockers (2). Taken together, the negative findings indicate that
the slow phase of catecholamine release does not originate from mobilization of intracellular Ca\(^{2+}\) via activation of muscarinic receptors. Electroporation was also considered as a mechanism contributing to the slow phase of release. High electric fields are known to cause transient pores in cell membranes (15, 36, 40). However, no evidence for electroporation was detected within slices using the tungsten-stimulating electrodes with the stimulation parameters used to observe the slow (or fast) phases of catecholamine release. It requires very large electric fields to cause electroporation of cells (at least 1 kV/cm). Given the intensity of the stimulation (2.45 V to achieve 350 \(\mu\)A) as well as the distance between the stimulating electrodes (100 \(\mu\)m), electric fields no larger than 250 V/cm were exposed to chromaffin cells within a slice. Furthermore, when electric fields close to 1 kV/cm were applied to the tungsten-stimulating electrodes, bubbles were observed at the electrode surface, indicating that electroporation using electrodes with a tungsten surface is not feasible because the electric fields required to cause electroporation also cause the breakdown of water at the electrode and would cause visible tissue damage.

Owing to the crucial contribution of Ca\(^{2+}\) signaling in the secretion of catecholamines (7, 8, 14), it is important to pay close attention to the types of VGCCs that are present on the cell being studied, as well as the different roles each plays in the multistep process of release. It has been shown that while N and P/Q VGCCs are functionally coupled with exocytosis on chromaffin cells and are believed to be spatially related to one another (3, 13), while L-type VGCCs, which take a longer time than P/Q and N VGCCs to inactivate, are known to regulate the autorhythmicity of chromaffin cell action potentials and could play a key role in the homeostatic mechanism of catecholamine release (25). However, L-type VGCCs are not present on splanchnic nerves but do play a role in direct stimulation of chromaffin cells by high K\(^+\) and therefore are an excellent candidate for investigating the difference between the two mechanisms (1, 23, 41). These properties of L-type VGCCs support the data observed here where the slow phase, not observed in stimulations exciting only the splanchnic nerve, is slower in onset and due to chromaffin cell excitation near the stimulating electrodes. Similar to results previously observed, the nicotinic acetylcholine receptor-mediated release decreased \(~25\%\) in the presence of nifedipine, while the slow phase was no longer distinguishable, leaving only the signal due to the decay of the fast phase (1). Similar to earlier work, the fast phase was highly dependent on extracellular calcium, further corroborating the splanchnic origin of this signal. As shown by Wakade and Wakade (50), completely depleting the extracellular buffer of calcium using EGTA abolishes catecholamine release elicited by direct depolarization of the chromaffin cells. While other calcium channels on the chromaffin cells could be contributing to the signal from direct chromaffin cell stimulation, it is apparent that the L-type channels play a key role in the entry of Ca\(^{2+}\) into the chromaffin cells during this process.

In this study, the high temporal and spatial resolution of FSCV at carbon fiber microelectrodes allowed us to distinguish between direct depolarization of chromaffin cells in murine adrenal slices and depolarization of splanchnic nerves that innervate them. While whole gland stimulations (24, 48, 50) established this difference, in this work we were able to temporally and spatially resolve the two components. It is well

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Fig. 7. Modulation of remotely stimulated release by ions. A: representative experiment showing the effect of 100 \(\mu\)M Hex with 200 \(\mu\)m between the stimulating and working electrode. B: representative experiment showing the effect of 1 \(\mu\)M TTX with 200 \(\mu\)m between the stimulating and working electrode. C: summary of effects of perfusion of Hex (\(n = 3\)) and TTX (\(n = 3\)) as well as the recovery of fast release when the stimulating electrode is 200 \(\mu\)m from the working electrode. Bar in A and B represents time of stimulation.***\(p < 0.001\).
known in the literature that care must be taken to select appropriate parameters for electrical stimulation of living tissue to study mechanisms of release (26). The present work shows that lower currents and smaller stimulation pulse widths are the proper choice when studying secretion from murine adrenal slices since they trigger the physiological mechanisms which lead to catecholamine release.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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