Electrophysiological and morphological features underlying neurotransmission efficacy at the splanchnic nerve-chromaffin cell synapse of bovine adrenal medulla

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de Diego AM. Electrophysiological and morphological features underlying neurotransmission efficacy at the splanchnic nerve-chromaffin cell synapse of bovine adrenal medulla. Am J Physiol Cell Physiol 298: C397–C405, 2010. First published November 25, 2009; doi:10.1152/ajpcell.00440.2009.—The ability of adrenal chromaffin cells to fast-release catecholamines relies on their capacity to fire action potentials (APs). However, little attention has been paid to the requirements needed to evoke the controlled firing of APs. Few data are available in rodents and none on the bovine chromaffin cell, a model extensively used by researchers. The aim of this work was to clarify this issue. Short puffs of acetylcholine (ACh) were fast perfused to current-clamped chromaffin cells and produced the firing of single APs. Based on the currents generated by such ACh applications and previous literature, current waveforms that efficiently elicited APs at frequencies up to 20 Hz were generated. Complex waveforms were also generated by adding simple waveforms with different delays; these waveforms aimed at modeling the stimulation patterns that a chromaffin cell would conceivably undergo upon strong synaptic stimulation. Cholinergic innervation was assessed using the acetylcholinesterase staining technique on the supposition that the innervation pattern is a determinant of the kind of stimuli a chromaffin cell would receive. A reliable method to produce frequency-controlled APs by applying defined current injection waveforms is achieved; 2) the APs thus generated have essentially the same features as those spontaneously emitted by the cell and those elicited by fast-ACh perfusion; 3) the higher frequencies attainable peak at around 30 Hz; and 4) the bovine adrenal medulla shows abundant cholinergic innervation, and chromaffin cells show strong acetylcholinesterase staining, consistent with a tight cholinergic presynaptic control of firing frequency.

bovine chromaffin cell; acetylcholine; acetylcholinesterase; action potential; patch clamp

THE RATE OF ADRENAL MEDULLA catecholamine release into the circulation must be precisely controlled. On the one hand, upon an emergency situation requiring a quick response, large amounts of catecholamines are released to the blood flow to prepare the body for action: bronchodilatation, pupil dilation, skeletal muscle vasodilatation, visceral vasoconstriction, digestion suppression, cardiac positive inotropic effects, mobilization of glycogen from the liver, or increased attention are among the effects of adrenaline (9). On the other hand, an excessive amount of circulating catecholamines can have fatal consequences, namely, cardiac arrhythmias. This balance is regulated by the sympathoadrenal axis. At the low firing rate of sympathetic nerves in resting conditions (~0.2 Hz (20)), the basal circulating levels of catecholamines help maintain the animal’s body homeostasis (“breed and feed response”). During an alarm situation, the firing of sympathetic nerves augments 10-fold or more to cause a sudden surge of circulating catecholamines, to prepare the organism for the “fight or flight” response. At these high frequencies, many quanta of acetylcholine (ACh) are released, stimulating the chromaffin cell membrane, which, in turn, fires action potentials (APs) at higher frequencies, releasing more catecholamine.

The works that use bovine chromaffin cells as a research model are counted by the hundreds. Yet little effort has been devoted to understanding their behavior with physiological stimuli. Cholinergic agonists have been applied for hundreds of milliseconds to study their relation to exocytosis (3, 21, 27, 55). Exocytosis and endocytosis elicited by trains of AP-like waveforms or trains of recorded APs have been studied with patch clamp in the voltage-clamp configuration, combined with capacitance and/or amperometry by the groups of Artalejo (24), Fox (17), Smith (11), Stuenkel (30), and our group (19) in bovine chromaffin cells, by Smith in mouse chromaffin cells (12, 28), and Zhou in rat chromaffin cells (23). Some studies have used the perfused adrenal gland technique stimulating sectioned branches of the splanchnic nerve to elicit secretion (53). However, except for one previous publication of our group (19), no available work looks into evoked AP excitability at the chromaffin cell level in the bovine species.

Considering the firing of APs as the main stimulus that produces controlled catecholamine release in the intact gland, a major aim of this study has been to determine what the requirements and limitations are for evoking frequency-controlled APs, also to determine whether the innervation pattern of the bovine adrenal medulla chromaffin cells is compatible with such kind of stimulation. This study is tackled by several approaches: fast intermittent perfusion of ACh, various electrical stimulation waveforms (ACh-like current ramps, superimposition of such waveforms, and square depolarizing pulses), and histochemical staining of acetylcholinesterase (AChE) to reveal the cholinergic innervation of chromaffin cells, as well as the spatial distribution of the enzyme in the medulla, on the supposition that the innervation pattern would greatly determine the kind of cholinergic stimuli chromaffin cells can receive.

METHODS

Cell culture. All experimental procedures were performed following the rules of and approved by the Universidad Autónoma medical school’s ethical committee for the care and use of animals in research,
in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC) and with the Spanish Real Decree of October 10, 2005 (RD 1201/2005). Adrenal glands from 12- to 18-mo-old cows were obtained from the city slaughterhouse under the supervision of the local veterinary service. Bovine chromaffin cells were isolated following standard methods (45). Cells were suspended in Dulbecco’s modified Eagle’s medium, supplemented with 7% fetal calf serum, 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Cells were plated on 13-mm-diameter glass coverslips at a density of 75 × 10⁴ cells/coverslip and were kept at 37°C in a water-saturated incubator, with a 5% CO₂/95% air atmosphere. Cells were used during days 1–4 in culture. 

Recordings of membrane potential with the patch-clamp technique. Membrane potential (E_m) variations were measured with the current-clamp configuration of the patch-clamp technique (33). The setup was composed of a Nikon Diaphot inverted microscope and an EPC-10 (HEKA Elektronik, Lambrecht, Germany) patch-clamp amplifier controlled by PULSE software running on a personal computer. Some patch-clamp amplifiers are unable to follow fast voltage changes (46), but the EPC-10 amplifier has this capability, allowing for “true” current-clamp recordings (EPC-10 manual). Patch pipettes were pulled from borosilicate glass (Kimble-Kontes) covered with dental wax and fire polished; pipette resistance was ~2 MΩ. To avoid washout of cytoplasm constituents, the perforated-patch mode (36, 44) of the patch-clamp technique was used. Amphoter cin B was the permeating agent (1, 56). Seals were achieved in a standard Tyrode solution composed of (in mM) the following: 137 NaCl, 1 MgCl₂, 2 CaCl₂, 5.3 KCl, 10 glucose, and 10 HEPES. The potential was held at ~70 mV, and the access resistance monitored until it decreased <20 MΩ. Then the amplifier was switched to the current-clamp mode, and current “injection” set to 0 pA. Only cells with a leak <5 pA at a holding potential of ~70 mV were used for experiments. Series resistance compensation of 95% was applied in some of the experiments. This slightly changed the timing of the APs, but did not seem to affect the frequency; besides, in some cells, the E_m became unstable. Hence it was decided not to apply this compensation procedure in the experiments.

Perfused waveforms were built with a custom macro written in IgorPro (Wavemetrics) following the directions of the HEKA manual. They were read by the HEKA Pulse software and applied by the amplifier. The pipette solution composition was (in mM) as follows: 140 KCl, 8 NaCl, 10 HEPES, 2 MgCl₂. Patch pipettes were tip-dipped in amphotericin B free solution to facilitate sealing and backfilled with solution containing 500–750 μg/ml amphotericin B.

Calibration of the perfusion system. A homemade perfusion system was used to deliver fast puffs of ACh to current-clamped chromaffin cells. External solutions were rapidly exchanged using a5 % CO₂/95% air atmosphere. Cells were used during days 1–4 in culture. 

Electrodes were made by pulling from borosilicate glass (Kimble-Kontes) covered with dental wax and fire polished; pipette resistance was ~2 MΩ. The setup was composed of a Nikon Diaphot inverted microscope and an EPC-10 (HEKA Elektronik, Lambrecht, Germany) patch-clamp amplifier controlled by PULSE software running on a personal computer. Some patch-clamp amplifiers are unable to follow fast voltage changes (46), but the EPC-10 amplifier has this capability, allowing for “true” current-clamp recordings (EPC-10 manual). Patch pipettes were pulled from borosilicate glass (Kimble-Kontes) covered with dental wax and fire polished; pipette resistance was ~2 MΩ. To avoid washout of cytoplasm constituents, the perforated-patch mode (36, 44) of the patch-clamp technique was used. Amphoter cin B was the permeating agent (1, 56). Seals were achieved in a standard Tyrode solution composed of (in mM) the following: 137 NaCl, 1 MgCl₂, 2 CaCl₂, 5.3 KCl, 10 glucose, and 10 HEPES. The potential was held at ~70 mV, and the access resistance monitored until it decreased <20 MΩ. Then the amplifier was switched to the current-clamp mode, and current “injection” set to 0 pA. Only cells with a leak <5 pA at a holding potential of ~70 mV were used for experiments. Series resistance compensation of 95% was applied in some of the experiments. This slightly changed the timing of the APs, but did not seem to affect the frequency; besides, in some cells, the E_m became unstable. Hence it was decided not to apply this compensation procedure in the experiments.

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Calibration of the perfusion system. A homemade perfusion system was used to deliver fast puffs of ACh to current-clamped chromaffin cells. External solutions were rapidly exchanged using electronically driven miniature solenoid valves coupled to a handmade four-barrel concentration-clamp device, the common outlet of which was placed within 50 μm of the cell. The flow rate was regulated by gravity. To assess its delivery and washout speed, the amperometry technique was used. As several puffs of the perfusion solution were applied, had we used open-tip patch pipettes, the solutions of different ionic composition (that in the pipette and the one perfused) would have mixed, yielding different amplitude currents with repeated stimulations. The use of amperometry electrodes aimed at obtaining stable currents when several puffs were applied, thus assessing the stability of the perfusion. Electrodes were built as previously described (40) and were 10 μm in diameter. A polarized (700 mV with respect to ground) carbon fiber electrode (CFE) was placed aside a chromaffin cell, and a 100 μM puff of norepinephrine (NE) was applied for 25 ms every 1 s. Good care was taken to place the CFE at the same distance of the perifusion system tip as the chromaffin cell would be in standard recording conditions. Since NE gets oxidized on touching the polarized CFE, it generates an electrical current that is recorded by the amplifier. We tested three CFEs, applying 8–20 puffs at 1 Hz to each one. We calculated rise time as well as total duration of each current (means shown are per CFE, not per puff). It took 18.9 ± 0.9 ms (mean ± SE, n = 3) to reach maximum current amplitude. Since current amplitude is proportional to the concentration of the substance being oxidized, this is the time it takes for the perifusion solution to reach its maximum concentration in the surroundings of the CFE tip. The half-width was 36.4 ± 5.9 ms, and it took 85.9 ± 8.2 ms (mean ± SE) to totally wash out since the initial response.

Histochemistry. Bovine adrenal glands were gently perfused with 50 ml of saline solution (NaCl 0.9%), followed by 50 ml of freshly prepared 4% parformaldehyde in 0.1 M phosphate buffer, pH 7.4; they were postfixed in the same fixative solution at 4°C and then cut in longitudinal as well as transversal 1- to 2-cm-thick slices. The slices were cryoprotected by immersion in 30% sucrose in 0.1 M phosphate buffer, pH 7.4, and were thereafter embedded in OCT (Sakura, Finetek) and frozen at −80°C. Then slices were cut into 20-μm sections with a cryotome (Leica CM 1950) and mounted on poly-lysine-treated slides (Polysine, Menzel and Company, Brunswick, Germany). The AChE staining was achieved following the method of Karnovsky and Roots (39) by immersing the sections for 30 min at 37°C in a buffered solution that contained iodized acetylthiocholine, which acts as substrate for AChE, potassium ferricyanide, and copper sulfate; the two latter chemicals react with the liberated thiocyanate, producing a brownish precipitate. Sections were then dehydrated, immersed in xylene, and mounted on coverslips with DePeX (BDH, Poole, UK).

Chemical products. All chemical products were from Sigma (Sigma-Aldrich, Madrid, Spain) except for fetal calf serum and penicillin/streptomycin, which were from PAA Laboratories (Haidmannweg, Pasching, Austria) and Gibco-Invitrogen (Paisley, UK), respectively.

Data analysis and statistics. Given that not all cells emitted the same number of APs, pooling all of them together would give more weight to the features of the APs of those cells that emit more APs; this would bias the statistical comparisons. To avoid this analysis artifact, a mean value for each AP parameter was calculated from all of the APs analyzed in each cell; thus single values per parameter and per cell were pooled and used for comparisons; this approach makes each cell have the same weight and produces reliable results (14). Data sets that fulfilled the assumptions for the application of parametric tests were compared by means of one-way ANOVA, followed by Holm Sidak post hoc test when appropriate; otherwise, we used the nonparametric Kruskal-Wallis repeated-measures one-way ANOVA, followed by Dunn’s test when appropriate. All statistical analysis was performed using SPSS 13 (SPSS, Chicago, IL), MS Excel (Microsoft, Redmond, WA), and IgorPro. Groups of data were considered statistically different if statistical tests resulted in P values < 0.05.

RESULTS

Spontaneous activity of bovine chromaffin cells. Spontaneous firing of APs has been reported in chromaffin cells of mouse (47), cow (26, 54, 60), rat (31, 35, 41, 42, 50), gerbil, and human (6). Here, spontaneous activity has been recorded to obtain APs without any manipulation and compare them with elicited APs. Spontaneous activity was recorded for 30–60 s before application of a stimulation protocol. Approximately 35% of the cells tested (18 of 52) fired APs spontaneously. Frequency of appearance was very irregular: some cells emitted APs, and human (6). Here, spontaneous activity has been recorded to obtain APs without any manipulation and compare them with elicited APs. Spontaneous activity was recorded for 30–60 s before application of a stimulation protocol. Approximately 35% of the cells tested (18 of 52) fired APs spontaneously. Frequency of appearance was very irregular: some cells emitted APs, and some did not. The frequency of APs was measured over a period of 30–60 s and expressed as the number of APs per second. The distribution of AP frequencies was skewed, and the mean frequency was calculated to compare different groups. The mean frequency of spontaneous AP firing was ~0.2 Hz.
Mean resting $E_m$ was $-51.9 \pm 1.3$ mV (52 cells from 12 different cultures). A kinetic analysis of the APs generated was performed, and the following features were extracted (see Fig. 1 legend, Fig. 1B): amplitude of the AP, rise time, half-width, decay time, posthyperpolarization amplitude, time to peak posthyperpolarization, and recovery time constant.

The kinetics of a minimum of 6 (up to 30) spontaneously occurring APs per cell fired by 7 cells were analyzed. Each kinetic parameter was averaged for all of the APs in each cell; a statistical summary is shown in Table 1. Spontaneously emitted APs were rather variable in amplitude, which ranged from a mean of 52 mV in one cell to 78 mV in another (mean $65.1 \pm 4.4$). Half-width was 2.7 ms, and rise time was $\sim 60$ ms. Usually the membrane depolarized slowly until the AP firing threshold was reached.

### Table 1. Features of the APs recorded under different stimulation conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Amplitude, pA</th>
<th>Rise Time, ms</th>
<th>Half-width, ms</th>
<th>Decay Time, ms</th>
<th>Posthyperpolarization Amplitude, pA</th>
<th>Time to Peak Posthyperpolarization, ms</th>
<th>Tau Recovery, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous (7, 98)</td>
<td>63.7 ± 3.7</td>
<td>57.1 ± 11.8</td>
<td>2.7 ± 0.4</td>
<td>2.7 ± 0.4‡</td>
<td>19.7 ± 1.5†</td>
<td>4.6 ± 0.7</td>
<td>32.7 ± 7.9</td>
</tr>
<tr>
<td>ACh (7, 151)</td>
<td>76.3 ± 4.2</td>
<td>38.0 ± 8.2</td>
<td>2.8 ± 0.2</td>
<td>4.1 ± 0.4</td>
<td>12.3 ± 1.2</td>
<td>3.9 ± 0.5</td>
<td>3.9 ± 0.5</td>
</tr>
<tr>
<td>Electrical (6, 280)</td>
<td>69.0 ± 3.6</td>
<td>13.3 ± 1.1†</td>
<td>2.7 ± 0.2</td>
<td>2.4 ± 0.2‡</td>
<td>19.5 ± 2.2</td>
<td>4.2 ± 0.2</td>
<td>24.0 ± 4</td>
</tr>
</tbody>
</table>

Values are means ± SE; nos. in parentheses are the no. of cells and the no. of action potentials (APs) analyzed, respectively. Recovery from undershoot to resting membrane potential was obscured in acetylcholine (ACh)-elicited APs by a hump, probably due to residual ACh. That is why tau recovery is not given for this group. The parameters measured were as follows: the amplitude of the AP and time to peak (rise time), from resting membrane potential to peak depolarization; the half-width, i.e., the width of the AP spike at the point halfway of peak depolarization; decay time, from peak to initial resting membrane potential; time to maximum posthyperpolarization (or undershoot) and minimum voltage reached, i.e., time to reach this peak hyperpolarized value from resting membrane potential (after the spike); finally, the recovery time constant, which was calculated by fitting a single-exponential equation from peak undershoot $\leq 100$ ms after it. The time constant of this curve was taken as an index of the return to basal velocity. *P < 0.05, †P < 0.01, with respect to ACh elicited APs. All comparisons are performed with parametric one-way ANOVA, except rise time and tau recovery, which did not fulfill parametric assumptions. A nonparametric ANOVA on ranks was applied in these cases.

### Fig. 1. Spontaneous and acetylcholine (ACh)-elicited action potentials (APs). A: original recording of spontaneous activity from a bovine chromaffin cell. One of these APs is shown at a larger time scale in B, together with the kinetic parameters analyzed: rise time (rt), amplitude from resting membrane potential ($E_m$) to maximum overshoot (amp), half-width (hw), decay time (dt), time from resting $E_m$ value (after the AP) to maximum undershoot (thip), and amplitude of the undershoot (hamp). Perfusion of short pulses of ACh induced the firing of a single AP per pulse. C, top: sample recording of such an ACh-elicited APs train at 0.3 Hz. C, bottom: effect of constant perifusion of ACh is shown. D: one of the ACh-elicited APs is shown at an extended time scale, along with the nicotinic current generated from a brief 25-ms ACh puff recorded in the same voltage-clamped cell.

**ACH-induced APs.** Splanchnic nerve stimulation releases ACh (25) that elicits APs in adrenomedullary chromaffin cells in situ (4, 34, 38). These APs open the voltage-gated Ca$^{2+}$ channels, letting Ca$^{2+}$ enter the cell and triggering the exocytotic process (22). This mechanism is supposed to finely control the release of catecholamines in the adult animal. In a previous work (20), we have tried to reproduce this twitching stimulation in vitro by perfusing 30 μM ACh for short times (25 ms) and recording the changes in the $E_m$ thus induced. It was possible to elicit the firing of a single AP with this kind of stimulation (20). Here, a more reliable, fast perifusion system has been built, and its speed calibrated with a CFE (see METHODS).

An ACh stimulus delivered in this perifusion system, applied in voltage-clamp conditions at a holding potential of $-50$ mV thus induced. It...
mV, similar to the resting chromaffin cell $E_m$, produced a mean inward current ($I_{ACh}$) of $\sim 340$ pA ($384.4 \pm 31.5$ pA, 4 cells) that reached its peak current in $\sim 24$ ms ($24.16 \pm 1.72$ ms, see Fig. 1D). This kind of ACh stimulus can be applied repeatedly to obtain APs at a desired frequency. We found that, at frequencies $\leq 1$ Hz, the method worked fine (Fig. 1C, top). Although the speed of the perfusion system suggests that frequencies up to 10 Hz can be achieved, in practice, with long trains at frequencies $> 3$ Hz, most APs began to fail, and the $E_m$ became unstable during the stimulation, although, on washout for a few seconds, the cell $E_m$ recovered stability. This is possibly due to not enough washing out of the ACh between two puff intervals at higher frequencies; ACh may accumulate for long periods of time in the bath and may activate depolarizing currents or cause desensitization of cholinergic receptors. This pulsatile stimulation dramatically contrasted with constant superfusion of 30 $\mu$M ACh (Fig. 1C, bottom). The membrane depolarized rapidly and three or four APs were fired, then the depolarization decayed to a stable plateau ($15.1 \pm 1.50$ mV over $E_m$, 8 cells) until washout, when the cell membrane rapidly hyperpolarized.

ACh-elicited APs were obtained from eight cells (276 APs). Only 52% of them showed a measurable posthyperpolarization, which mean amplitude (from initial resting potential until peak hyperpolarizing value after the AP) was $13.1 \pm 1$ mV. Kinetic analysis was carried out only on these APs (see Table 1). They had mean amplitude of $\sim 76$ mV. Their half-width was 2.8 ms, and rise time took 38 ms. The low percentage of APs with posthyperpolarization observed is due to a not fast enough washout of the ACh, which is still exerting its effect; this effect can be observed in a humplike depolarization found after the AP in most events induced this way (Fig. 1D, arrow). It is interesting to notice though, that this humplike depolarization has been described in guinea pig chromaffin cells in situ strongly stimulated by endogenous ACh (34), as well as in other cell types, such as sympathetic neurons of the guinea pig celiac ganglion (48), indicating that a massive ACh released at the synapse may “overflow” the washout mechanisms. Placing the perfusion pipette tip in the right position close to the cell was critical to obtain good responses.

AP induction by the application of current waveform patterns. Based on the kinetics of the $I_{ACh}$ described in the previous epigraph and in the ones found in the literature, we built current injection protocols using ramps that were delivered by the amplifier. Large-amplitude $I_{ACh}$, such as the one described in the previous section, which reached $> 300$ pA, would ensure the firing of an AP. However, it seemed to be a much larger stimulus than what would be needed to depolarize the cell to a level just enough to reach AP firing threshold. Barbara and Takeda (5) and Kajiwara et al. (38), working with rat adrenal slices, found that the quantal size of the ACh released by the splanchnic nerve, measured postsynaptically as an excitatory postsynaptic currents (EPSC) in the chromaffin cell, is in the order of 20–30 pA. Rise times are in the order of 2–3 ms, and decay time constants are $\sim 10$ ms. Assuming similar ACh quantal characteristics for the bovine species, it was found that the following current injection waveform elicited APs with high probability: a ramp from 0 to 30 pA in 4 ms, from 30 to 10 pA in 5 ms, and from 10 to 0 pA in 3 ms (Fig. 2A). This stimulus applied at 1 Hz elicited single AP firing in 93% of 300 trials applied to six cells.

The kinetics of the APs generated was compared with ACh perfusion-elicited and spontaneous AP firing. Rise times were faster than both, the spontaneous and the ACh-elicited APs. Decay times were faster, and posthyperpolarization amplitudes larger, than in the ACh-elicited APs, likely due to not enough ACh washout, as has been suggested in a previous epigraph. It
is worth noting that half-widths were similar in all three experimental situations (spontaneous AP emission, ACh elicited and electrically induced), pointing to an “all or nothing” response once firing threshold has been reached.

On the different AP firing frequencies attainable. To attain higher frequencies of stimulation, several strategies were tried. A first approach was to repeat the current waveform described in the previous section at different frequencies. This way, AP firing rates up to 5 Hz could be obtained. Figure 2B shows an example train of 50 APs elicited at 3 Hz. At higher frequencies, many stimulus waveforms failed to elicit APs (Fig. 2C). However, by increasing the maximum amplitude while keeping the timing of the waveform equal, higher frequencies could easily be obtained. The maximum amplitude of the current waveform was incremented in 30-pA steps, on the grounds of previous studies on rat splanchnic-chromaffin synapse EPSCs (5). It has also been shown that one to four splanchnic fibers make synapse with every rat chromaffin cell (38); a similar figure can be observed in the bovine adrenal medulla in this study (see Fig. 4). It is a conceivable scenario that simultaneous release occurs from two, three, or even four fibers, resulting in approximately proportional increments of this “elemental” EPSC. When peak current of the example cell in Fig. 2 was raised from 30 pA (which resulted in failure to induce APs in about one-half of the applications, Fig. 2C) to 60 pA (Fig. 2D), all stimuli produced successful AP firing.

To sum up, in six cells, a train of 50 waveforms peaking at 30 pA applied at 5 Hz resulted in the firing of a mean of 32 ± 6 APs, while incrementing the maximum of the first segment of the waveform to 60 pA resulted in a mean firing of 48 ± 1 APs out of 50. In five cells stimulated at 10 Hz, peak 30-pA waveforms induced an average firing of 22 ± 6 APs out of 50 stimuli, while peak 60 pA waveforms elicited 48 ± 2 APs. At 20 Hz, trains of peak 30-pA waveforms produced low success rate, but in seven cells stimulated with the peak 60-pA current waveforms, 40 ± 4 APs out of 50 stimuli were obtained. Thus with this kind of stimulation, efficient AP firing can be obtained with frequencies as high as 20 Hz.

ACh “quanta” could be released with some overlap. To test how this kind of stimulation would affect AP firing, protocols composed of 10 simple current waveforms were built and applied with several time delays. Thus, with zero delay, all current waveforms are added simultaneously; with 1-ms delay, one current waveform onset occurs 1 ms after the previous had begun; with 2-ms delay, one current waveform onset occurs 2 ms after the previous one; and so on. In Fig. 3, A and B, the current waveforms applied for 2- and 7-ms delays, respectively, are shown with the responses generated. Five cells from two different cultures were challenged with this protocol one to four times. None of the delays, from total overlap (0-ms delay) to total contiguity (12-ms delay), produced more than four APs. Three of them produced just one to two APs, no matter the waveform applied, while others showed AP firing along the whole stimulation period. In the latter cells, the number of APs generated was lower at short delays, incrementing from one to two APs at 0–6 ms, to two to three APs at 6–11 ms, and three to four APs at 11–12 ms delay. It should be noted that the last APs were reduced in amplitude with respect to the first. Considering the bovine chromaffin cells that generated more responses, cell AP firing frequency would top at ~30–35 Hz.

A common manner of eliciting AP firing in chromaffin cells (26, 34, 38) and in other excitable cells (10, 15, 37, 48, 62) is the use of constant-current injections. Hundreds of millisecond long step current injections were tested to try to produce barrages of higher frequency APs. Cells were challenged with either 200- or 500-ms duration pulses. Of 10 cells tested, only 1 fired continuously during the whole length of the stimulation.

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**Fig. 3.** APs elicited by overlapped waveform and square current injections. Ten ACh-based current waveforms were overlapped with variable delays between them (0–12 ms). A: the 2-ms delay overlap waveform (shaded line) and its response (solid line). B: the 7-ms delay overlap waveform and its response are depicted. Constant-current injection waveforms were also tested. C: in most cells, 1–3 APs were fired at the beginning of the stimulus, and the remaining time the membrane remained depolarized to a level proportional to the intensity of the stimulus. D: in few cells, short stimuli of a few hundreds of milliseconds were able to elicit a longer burst of APs, but, when the stimulus was lengthened, the burst stopped as in the other type.
period (200 ms, 6 APs, Fig. 3D); this is again a frequency limit of some 30–35 Hz. With a 500-ms stimulation period, none of the seven cells challenged was able to fire APs during the whole length of the current injection. One of them fired six APs in the first 200–300 ms, and the rest one to five APs. Current injections of 5, 10, or 20 pA were also tested to try to depolarize the cell just to firing threshold and achieve an oscillating pattern of APs. Alternatively, constant-current injections of 60 or 90 pA were also tried. None of these stimuli produced a qualitatively different outcome. Thus most bovine chromaffin cells responded with one or a few APs at the beginning of the pulse, and then the cell remained in a depolarized level until the end of the pulse (See Fig. 3C).

**Bovine adrenal medulla cholinergic innervation.** Longitudinal and transversal slices of the bovine adrenal gland were stained with a cholinesterase staining technique to assess the cholinergic innervation (39). Figure 4, A and B, shows strong AChE activity localized in the medulla, whereas the cortex shows no staining. Other sections were immunostained with an antibody raised against tyrosine hydroxilase, an enzyme present in all chromaffin cells, since it is necessary for the synthesis of catecholamines. A similar spatial localization of the chromaffin tissue in the medulla of the gland was found (data not shown).

Upon larger magnification, some loose nerve fibers could be identified in the cortex in areas close to the medulla; some scarce thin fibers embedded in connective tissue traveled from the capsule all the way to the medulla. The transversal sections showed thick nerve tracts in the central medullary area composed of bundles of cholinergic axons that appeared as densely marked sets of points as they were transversally cut (Fig. 4C). In the longitudinal sections, nerve tracts in the medullary area were longitudinally cut, and nerve bundles could be observed embedded in connective tissue (Fig. 4D). Cholinergic fibers form plexuses that wind round chromaffin cell acini, suggesting the existence of boutons “en passant.” Numerous terminal boutons can be observed as black dots at fiber endings (Fig. 4, E–H).

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**Fig. 4.** Cholinergic innervation of the adrenal gland revealed by acetylcholinesterase (AChE) staining. AChE was stained in transversal (A, C, E, and G) and longitudinal (B, D, F, and H) sections of a paraformaldehyde-fixed adrenal gland. Microphotographs are bright field. A and B: the whole slices are shown. On the top left corner, a bovine adrenal gland silhouette shows the area where the section was cut with a dashed line. The longitudinal section was off-centered on purpose to avoid the large adrenal vein (AV) and obtain more proportion of medulla. C and D: low-magnification microphotographs of the medulla. Notice the densely stained nerve tracts (NT), which were abundant in the central part of the medulla in the transversal section. NT were cut longitudinally in the other orientation (D). E and F: at middle magnification, profuse nerve fibers (NF) can be observed traversing the medulla and ending in synaptic boutons on chromaffin cells (CC). E shows a rather fortunate field in the transversal section: a bundle of cholinergic fibers leave a nerve tract in the top right area, going to the opposite side of the field, innervating numerous CC along the way. G and H: the synaptic boutons are better appreciated at higher magnification (arrows). AC, acinus; CT, connective tissue; CX, adrenal cortex; M, adrenal medulla; V, vessel.
Chromaffin cells showed a diffuse cytoplasmic staining, likely due to intracellular or cell membrane AChE activity. This is consequent with previous works showing different AChE isoforms in membrane fractions (49) and intracellular organelles, such as the endoplasmic reticulum and Golgi apparatus (7).

**DISCUSSION**

In this study, isolated current-clamped chromaffin cells were stimulated with short millisecond ACh puffs, which are similar stimuli to those chromaffin cells likely undergo in their natural environment at the intact adrenal gland. ACh, the traditionally considered main adrenal secretagogue, has been used to induce AP firing in chromaffin cells. In trying to apply similar stimulation patterns to those possibly found at the synapse, current waveforms were created that were based on the kinetics of the inward currents elicited by ACh puffs and on the kinetics of previously published EPSCs obtained in chromaffin cells of rat adrenal slices (5). Current injections using these waveforms served to elicit APs at different frequencies in a predictable, computer-controlled manner up to 20 Hz. Current protocols built from overlapped single ACh-like waveforms were also applied with the aim of obtaining the highest AP frequency possible under conceivably physiological situations. Long constant-current injections were also tested. Finally, since it is likely that the pattern of innervation partially determines the kind of stimuli a chromaffin cell receives, cholinergic afferences to the bovine adrenal gland were studied by means of AChE staining.

Despite being considered the physiological neurotransmitter at the splanchnic nerve-chromaffin cell synapse in the adrenal medulla (25), only a few studies, none of them in the bovine species, have been performed with ACh to see its effects on spontaneous APs or on APs evoked by the neurotransmitter (6, 8, 41, 52). It is evident that these protocols do not resemble the pulsatile stimulation likely found in the gland, because the frequency of APs produced by ACh cannot be experimentally controlled in a situation where continuous exposure to ACh (and to other nicotinic agonists) leads to desensitization of nicotinic receptors (18, 29). The drawbacks of the ACh stimulation patterns referred to above are not present in the protocol of intermittent application of ACh puffs reported here. With this protocol, the frequency of APs elicited by ACh could be controlled at the experimenter’s will in the range of 0.2–3 Hz. It is interesting that this stimulation pattern resembles the firing rate of sympathetic-splanchnic nerve discharge under animal and human resting conditions (0.2 Hz) and in stressful conditions (2 Hz and above) (20).

With ramp current waveforms, kinetically similar APs and efficient firing at frequencies as high as 20 Hz were achieved. Given the fact that rise time, decay time, and time to peak posthyperpolarization add to ~18–20 ms in the fastest APs recorded, a top frequency of some 50 Hz or a little more could be reached. However, because the time to go back to resting $E_m$ levels, letting the membrane passively recover, is several tens of milliseconds, a more or less constant depolarizing stimulus must be applied to reach these high frequencies. When overlapping waveforms with several delays were applied or in some instances of constant current injections, the fastest frequency reached was some 30 Hz. These data suggest that the membrane excitability properties of bovine chromaffin cells are designed to fast regulate their firing rate, effectively refraining the release of a life-endangering amount of catecholamines to the circulation on the off chance of a massive cholinergic stimulation. In a previous report, a bell-shaped curve related the frequency of splanchnic nerve stimulation with the catecholamine secretion from cat adrenals (2); secretion peaked at ~8 Hz and then diminished with increasing stimulation frequencies. In the present study, we show that a possible explanation for this curve is that chromaffin cells reach their maximum frequency of AP firing; at the higher frequencies, the $E_m$ remains unable to fire APs in a somewhat depolarized level that produces a less effective secretion.

It is interesting that, when stimulated with long constant-current steps or with cluttered ACh-like waveforms, most cells produced only a few APs at the beginning of the stimulus. This behavior resembles the phasic firing of certain neurons (10, 48). Holman et al. (34) have described a similar pattern in rat chromaffin cells in situ in the adrenal slice preparation. They suggested that rat chromaffin cells fire APs in a phasic fashion. The results found in the present work suggest that these cells would demand a pulsatile pattern to transfer the presynaptic stimulus into higher AP firing frequencies (and into a greater ensuing secretion of catecholamines). Gap-junction coupling of chromaffin cells (13) could aid to synchronize the firing patterns of chromaffin cells and to achieve a simultaneous release from many cells in an acinus at the same time.

AChE is present at cholinergic synapses to ensure a rapid termination of the stimulus. In chromaffin cells, AChE has been reported to exist in the cell membrane and in different subcellular organelles (7, 49). Bovine chromaffin cells also secrete AChE upon depolarizing stimulation and in the presence of $Ca^{2+}$, suggesting an exocytotic mechanism, and thus the presence of the enzyme in some kind of secretory vesicle or endosome (51, 58). However, microphotographs of an AChE-stained bovine adrenal section have not been previously published. It was decided to perform this task, considering the pattern of innervation as one of the determinants of the kind of stimuli the postsynaptic cell can receive. The almost complete lack of cholinergic fibers in the bovine adrenal cortex, together with the orientation of the nerve tracts in the longitudinal and transversal sections, suggest a particular organization of the adrenal innervation: nerve tracts would enter through some central area, traversing the gland beside the vessels around the adrenal vein; then they would separate from the central bundles to innervate the chromaffin tissue. Figure 4E, in particular, shows nerve fibers leaving a transversally cut nerve tract to innervate several chromaffin cells, giving support to this hypothesis. The abundance of AChE in the bovine adrenal medulla suggests the following conclusions: 1) chromaffin cells are well endowed with profuse cholinergic innervation, as shown by the nerve fibers staining; and 2) the importance of a rapid termination of the ACh stimulation, suggested by the chromaffin cell staining and also by the AChE secretion described elsewhere. Both of these conclusions support the idea of a tight control of bovine chromaffin cell cholinergic excitability and, therefore, of cholinergically evoked catecholamine secretion.

It is plausible that, as in many cholinergic synapses, including the muscle end-plate (39a), the release of ACh quanta is pulsatile, thereby causing intermittent stimulation of nicotinic
receptors at the synapse formed by a splanchnic nerve terminal on a chromaffin cell at the adrenal gland. This pulsating stimulation pattern would be favored by rapid degradation of ACh by AChE present in the synapse, thus preventing desensitization of nicotinic receptors (61), and ensuring the efficacy of synaptic transmission and catecholamine release. In the case of a massive splanchnic discharge, the biophysical limits to AP firing, together with the fast action of the AChE, ubiquitous in the medulla, would protect the heart against a hazardous rise in circulating catecholamine levels.

Besides ACh, splanchnic terminals store a variety of substances, such as opioids (57), pituitary adenylate cyclase activating peptide (PACAP) (32), or vasoactive intestinal polypeptide (59). These molecules may act either as modulators of the chromaffin cell electrical activity and secretion (16), or as cotransmitters along with ACh (43, 59). A recent study using the mouse slice preparation shows that PACAP is of paramount importance in catecholamine secretion at higher frequencies of stimulation of the splanchnic nerve (43). With this stimulation pattern, it would take over the catecholamine secretion. Thus it will be of interest to study the contribution of these substances to cell excitability and secretion with the protocols developed in the present article. For instance, the fast perfusion system could be used to deliver pulsatile quantities of these modulators at different frequencies, with the advantage of controlling the timing of application and concentration of the substance. The effect of these molecules could also be tested on AP kinetics or AP-induced secretion with the electrically evoked AP protocols.

As a final methodological note, it is underlined that the ability to elicit frequency-controlled APs, either with the use of the stimulation pattern based in fast ACh puffs, or with current waveforms, combined with other techniques, such as amperometry and fluorescent imaging, should provide new information on the regulation by various factors, conditions, and ligands of the different splanchnic transmitter-mediated chromaffin cell responses, Ca\(^{2+}\) signals, exocytosis, and endocytosis.

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DISCLOSURES

I am not aware of financial conflict(s) with the subject matter or materials discussed in this manuscript with any of the authors, or any of the authors’ academic institutions or employers.

REFERENCES


