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Sawhorse Waveform Voltammetry for Selective Detection of Adenosine, ATP, and Hydrogen Peroxide

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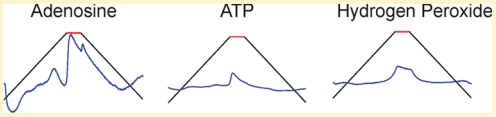
Supporting Information

ABSTRACT: Fast-scan cyclic voltammetry (FSCV) is an electrochemistry technique which allows subsecond detection of neurotransmitters *in vivo*. Adenosine detection using FSCV has become increasingly popular but can be difficult because of interfering agents which oxidize at or near the same potential as adenosine. Triangle shaped waveforms are traditionally used for FSCV, but modified waveforms have been introduced to maximize analyte sensitivity and provide stability at high scan rates. Here, a modified sawhorse waveform was used to maximize the time for adenosine oxidation and to manipulate the shapes of cyclic voltammograms (CVs) of analytes which oxidize at the switching potential. The optimized waveform consists of scanning at 400 V/s from −0.4 to 1.35 V and holding briefly for 1.0 ms followed by a ramp back down to −0.4 V. This waveform allows the use of a lower switching potential for adenosine detection. Hydrogen peroxide and ATP also oxidize at the switching potential and can interfere with adenosine measurements *in vivo*; however, their CVs were altered with the sawhorse waveform and they could be distinguished from adenosine. Principal component analysis (PCA) was used to determine that the sawhorse waveform was better than the triangle waveform at discriminating between adenosine, hydrogen peroxide, and ATP. In slices, mechanically evoked adenosine was identified with PCA and changes in the ratio of ATP to adenosine were observed after manipulation of ATP metabolism by POM-1. The sawhorse waveform is useful for adenosine, hydrogen peroxide, and ATP discrimination and will facilitate more confident measurements of these analytes *in vivo*.



Fast scan cyclic voltammetry (FSCV) is an electrochemical technique which allows subsecond measurements of neurotransmitters *in vivo*.[1−3] Traditional FSCV uses a triangular shaped waveform which is applied to a carbon-fiber microelectrode at a scan rate of 300−400 V/s.[4,5] Most FSCV research has focused on studying dopamine dynamics in the brain,[6−9] where dopamine is detected at 0.6 V and the waveform traditionally scanned to 1.0 V.[10] However, when the waveform is extended to 1.3 V, dopamine oxidative current increases[11] due to increased adsorption from oxygen functional groups and surface renewal from breaking carbon−carbon bonds on the surface.[12] With higher scan rates up to 2400 V/s, a sawhorse shaped waveform was implemented that holds at a 1.3 V switching potential for a half a millisecond.[13] The purpose of holding at 1.3 V was to stabilize and renew the electrode surface and not to allow more time for dopamine oxidation, as the surface adsorbed dopamine completely oxidized before the hold time. Waveform optimization has proven to be an important tool for maximizing analyte sensitivity[12] and to reduce fouling[1,14] at the electrode.

FSCV has also been used to measure several other important but more electrochemically challenging neurochemicals in the brain such as serotonin,[1] hydrogen peroxide,[15] and adenosine.[16] Adenosine poses a specific challenge due to its relatively high E^0 (~1.30 V),[17] so a switching potential of 1.45−1.50 V is necessary with FSCV.[16,18,19] Adenosine is a neuromodulatory molecule found in the brain[20−22] and is neuroprotective during conditions of ischemia[23,24] and hypoxia.[25,26] Detection of adenosine using FSCV[3,16,18] is beneficial for understanding how adenosine functions on the subsecond to second time scale.[27] A secondary peak has been observed with FSCV for adenosine detection that can aid in distinguishing the analyte; however, the secondary peak is harder to identify at very low concentrations.[18,28]

Other analytes have cyclic voltammograms (CVs) with peaks around the same potential as adenosine, including ATP and hydrogen peroxide, that can interfere with adenosine detection.[15,16,18] ATP can be released by exocytosis and then metabolized extracellularly to adenosine.[21] ATP and adenosine have the same electroactive adenine moiety[17] and their CVs are almost identical. However, FSCV detection of adenosine at carbon-fiber microelectrodes is 3−6 times more sensitive than for ATP, due to the negative charge of ATP.[18] Unlike adenosine and ATP, hydrogen peroxide does not have a secondary peak but the relatively slow kinetics of hydrogen peroxide mean that the main peak is detected at a similar potential as the primary peak for adenosine.[15,15,29,30] While scanning to higher potentials might help separate adenosine and hydrogen peroxide, this solution is not practical due to water hydrolysis. Thus, a waveform is needed which would allow for better discrimination between these analytes that does not require a higher switching potential.

In this study, we used a modified sawhorse waveform to discriminate between adenosine, ATP, and hydrogen peroxide. Holding the electrode at the switching potential allows more

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time for oxidation to occur without the need for a higher switching potential. We found that holding the electrode at the switching potential for 1.0 ms is sufficient to lower the oxidizing potential used for adenosine detection. Higher amounts of current were observed for adenosine with a 1.35 V switching potential at the sawhorse waveform compared to the triangle waveform. Holding for 1.0 ms at the switching potential produced an extra peak in the adenosine CV which was not present for hydrogen peroxide; thus, the two compounds could be distinguished from one another. Principal component analysis (PCA) was used to discriminate between adenosine, ATP, hydrogen peroxide, and dopamine, and the sawhorse waveform was better for distinguishing between the analytes. Mechanically stimulated adenosine in slices was accurately predicted as adenosine using the sawhorse waveform. Overall, adenosine can be detected with higher sensitivity and selectivity at lower potentials with the sawhorse waveform.

METHODS

Chemicals. Adenosine and dopamine standards were purchased from Sigma-Aldrich (St. Louis, MO) and ATP was purchased from Tocris Biosciences (Bristol, United Kingdom) and dissolved in 0.1 M $HClO_4$ for 10 mM stock solutions and diluted daily in Tris buffer for testing. Hydrogen peroxide (30 %) was purchased from Macron Fine Chemicals (Center Valley, PA) and diluted daily in Tris buffer to its final concentration. The Tris buffer solution consists of 15 mM Tris(hydroxymethyl)aminomethane, 1.25 mM NaH_2PO_4, 2.0 mM Na_2SO_4, 3.25 mM KCl, 140 mM NaCl, 1.2 mM $CaCl_2$ dehydrate, and 1.2 mM $MgCl_2$ hexahydrate at pH 7.4 (all Fisher, Suwanee, GA). For slice experiments, calibrations and training set solutions were performed in artificial cerebral spinal fluid (aCSF): 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 2.4 mM $CaCl_2$ dehydrate, 1.2 mM $MgCl_2$ hexahydrate, 25 mM $NaHCO_3$, 11 mM glucose, and 15 mM tris(hydroxymethyl) aminomethane, pH 7.4 (all Fisher, Suwanne GA). POM-1 (sodium polyoxotungstate), an NTPDase 1,2 and 3 inhibitor, was purchased from Tocris. All aqueous solutions were made with deionized water (Milli-Q Biocel, Millipore, Billerica, MA).

Carbon-Fiber Microelectrodes. Carbon-fiber microelectrodes were fabricated from T-650 carbon-fibers (gift from Cytec Engineering Materials, West Patterson, NJ)[31] and cylinder-shaped electrodes, approximately 50−100 μm long, were used. Electrodes were sealed with Epon Resin 828 (Miller-Stephenson, Danbury, CT) and cured with 14% (w/w) 1,3-phenylenediamine hardener (Sigma-Aldrich, St. Louis, MO). All electrodes were soaked for 10 min in isopropanol (Fisher Scientific) prior to use.

Electrochemistry Measurements. Fast-scan cyclic voltammograms were collected using a ChemClamp (Dagan, Minneapolis, MN), and data was collected using Tarheel CV software (gift of Mark Wightman, UNC) using a home-built data analysis system and two computer interface boards (National Instruments PCI 6052 and PCI 6711, Austin, TX). The electrode was scanned from −0.4 to 1.45 V (vs Ag/AgCl) and back with a scan rate of 400 V/s and a repetition rate of 10 Hz for the triangle waveform. For the sawhorse waveform, the electrode was scanned from −0.4 to 1.35 V and held for 1.0 ms before ramping back down, at a scan rate of 400 V/s and 10 Hz repetition rate.

Brain Slice Experiments. Male Sprague−Dawley rats (250−350 g, Charles River, Willmington, MA) were housed in a vivarium and given food and water *ab libitum*. All experiments were approved by the Animal Care and Use Committee of the University of Virginia. Rats were anesthetized with isoflurane (1 mL/100 g rat weight) in a desiccator prior to slice preparation and were immediately beheaded. The brain was removed within 2 min and placed in 0−5 °C aCSF for 2−4 min for recovery. A vibratome (LeicaVT1000S, Bannockburn, IL) was used to prepare 400 μm slices of the prefrontal cortex. Slices were transferred to oxygenated aCSF (95% oxygen, 5% CO_2) and allowed to recover for approximately an hour before the experiment. aCSF (35−37 °C, maintained by an IsoTemp 205 water bath, Fisher Scientific) flowed over the brain slice at 2 mL/min. The electrodes were inserted 50 μm beneath the tissue, and the waveform was applied for 20 min before data collection. The slice was mechanically stimulated by using a micromanipulator to lower a glass pipet 50 μm. The pipet was ∼15 μm in diameter and located about 30 μm away from the working electrode. A PCA training set was collected by pressure ejection of adenosine, hydrogen peroxide, and ATP onto brain slices by a Parker Hannifin picospritzer (Picospritzer III, Cleveland, OH). The pipet was 30−50 μm from the carbon-fiber microelectrode. The ejection parameters were 10 psi for 100−400 ms and 100−800 nL of analyte (either 25 μM adenosine, ATP, or H_2O_2) was delivered to generate a training set in slices. All training sets were collected after mechanical stimulation.

For the POM-1 experiment, a training set of adenosine and ATP was generated followed by applying a mixture of 25 μM adenosine and 25 μM ATP. POM-1 (100 μM) in oxygenated aCSF was flowed over the slice for 30 min, and the mixture was applied again.

Principal Component Analysis. Principal component analysis software was written in LabView Mathscript RT Module (from Mark Wightman and Richard Keithley, UNC Chapel Hill). A training set was compiled for each analyte tested (adenosine, ATP, hydrogen peroxide, and dopamine). Principal components were extracted from the training set and the data was analyzed using principal component regression.[32] Mixtures of known concentrations of adenosine with hydrogen peroxide, ATP, or dopamine were analyzed. Every training set has residuals which account for currents from unknown signals, such as noise.[33] The Q-score is the sum of squares of the residuals for each variable. This was calculated, and any signal above Q failed and was not used in the analysis. In slices, a training set was generated by applying adenosine, hydrogen peroxide, and ATP in the brain slice.

Statistics. All values are reported as the mean ± standard error of the mean (SEM). All statistics were performed in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) and considered significant at the 95% confidence level ($p < 0.05$). One-way ANOVA with Bonferroni post tests were used to analyze the switching potential optimization and plateau time optimization experiments. Unpaired t tests were used to compare the currents at the switching potentials for the triangle and sawhorse waveform. Unpaired t tests were also used to compare adenosine concentration predicted by PCA to the actual value for mechanically stimulated adenosine.

RESULTS AND DISCUSSION

Comparison of the Triangle and Sawhorse Waveform. Cyclic voltammograms (CVs) for adenosine, ATP, and hydrogen peroxide have similar features at the traditional adenosine triangle waveform. Figure 1A shows background-subtracted current versus waveform time plots for 5 μM

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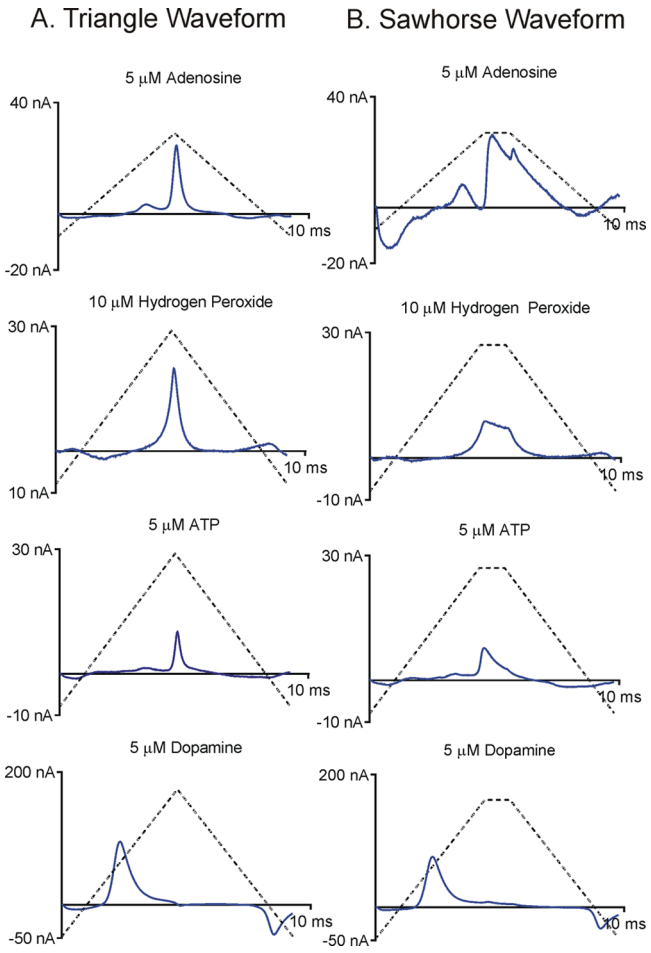

Figure 1. Current versus waveform time plots for the (A) triangle and (B) sawhorse waveform. The triangle waveform is the traditional adenosine waveform for FSCV (−0.4 to 1.45 V and back at 400 V/s). The optimized sawhorse waveform is scanning from −0.4 to 1.35 V, holding for 1.0 ms, and ramping back down to −0.4 V at a rate of 400 V/s. The data were collected at two separate electrodes. The dotted black line shows the shape of the waveform over time and the blue line represents the current vs applied waveform time. Data are plotted as current versus time instead of voltage because of the hold time in the sawhorse waveform; 5 μM adenosine, 10 μM hydrogen peroxide, 5 μM ATP, and 5 μM dopamine were tested.

adenosine, 10 μM hydrogen peroxide, 5 μM ATP, and 5 μM dopamine using the triangle waveform (−0.4 to 1.45 V and back at 400 V/s). The waveform trace is plotted on each of the plots (black dotted line) so that peak positions during the waveform can be analyzed. The main oxidation peak for adenosine, hydrogen peroxide, and ATP is right at, or slightly after, the switching potential. Adenosine has a secondary peak that appears around 1.0 V, which is more prominent at higher concentrations. ATP has the same oxidation reaction as adenosine, and the traces are similar except that carbon-fiber microelectrodes are more sensitive to adenosine.[16,18,19] Dopamine has a peak at 0.6 V and is shown as a control for comparison purposes.[34−36]

Keithley et al. first introduced the idea of a sawhorse waveform for dopamine detection using FSCV,[13] but the purpose of the sawhorse was to enhance electrode stability at scan rates exceeding 2000 V/s. Here, a modified sawhorse waveform was used to allow more time for analyte oxidation at the switching potential. Figure 1B shows background-subtracted current versus waveform time plots for the sawhorse waveform which scans from −0.4 to 1.35 V, holds for 1.0 ms, and then scans back down to −0.4 at 400 V/s. The current versus waveform time plots for the sawhorse waveform are from a different electrode than the triangle waveform plots because scanning to a higher potential can irreversibly change the electrode surface.[12] Traditionally, CVs are plotted as current versus voltage but due to the voltage plateau in the sawhorse waveform, the data are better visualized as a plot of current vs applied waveform time. The waveform is also superimposed on each plot in Figure 1.

Analytes which oxidize at the switching potential (adenosine, ATP, and hydrogen peroxide) look similar at the triangle waveform; however, at the sawhorse waveform the analytes are more distinguishable. The first difference between the plots from the sawhorse and triangle waveform is during the holding time. The background charging current decays during the holding potential (Figure 2A,B) due to the exponential decay in

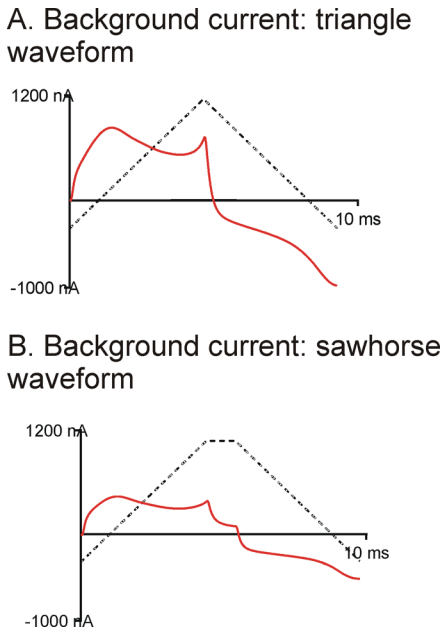

Figure 2. Background current for both waveforms. (A) Background current for the traditional triangle waveform (−0.4 to 1.45 at 400 V/s) is plotted in red and the black dashed line is the shape of the waveform over time. (B) Background current for the optimized sawhorse waveform (−0.4 to 1.35 V, hold for 1.0 ms at 400 V/s) is plotted in red and the black line denotes the shape of the sawhorse waveform over time. The sawhorse background current shows a drop in capacitive current at the plateau time.

capacitive charging. The faradaic current in the background subtracted current versus waveform time plot also decreases. For adsorption controlled species, the current will return to zero when all of the surface adsorbed species is oxidized. For diffusion controlled species, the current decays much slower. H_2O_2 is diffusion controlled (Figure S-1 in the Supporting Information) and its current falls off slowly with time during the holding potential (Figure 1B). Log plots of current vs time show a significantly slower rate of decay for hydrogen peroxide than for adenosine and ATP (Figure S-2 in the Supporting Information). Adenosine is primarily adsorption controlled,[16] and its current drops off faster at the switching potential than the current for hydrogen peroxide. ATP is also adsorption

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controlled (Figure S-1 in the Supporting Information) and because less is adsorbed than adenosine, the signal is back to zero at the end of the holding potential even though the rate of decay is similar to that for adenosine (Figure S-2 in the Supporting Information). Dopamine has no peak at the plateau because all the surface adsorbed dopamine is oxidized before that time.

Upon ramping back down, an extra peak for adenosine occurs in the sawhorse waveform. The extra peak is likely due to a background change after adenosine adsorption. The adsorption of a species to the electrode changes the background charging current due to differences in surface area or exposed surface oxide groups. Previous studies suggests that scanning to high anodic potentials causes electrode surface renewal due to breaking of carbon−carbon bonds.[12] If the surface was completely renewed on each scan, you would not expect a subsequent adsorption peak upon ramping back down. However, it is unclear how long the electrode needs to be held at high potentials in order to completely renew the surface. In the previous report, complete surface renewal occurred after 15 min of electrode cycling to high potentials;[12] therefore, it is unlikely that the electrode surface would be completely renewed after 1.0 ms of holding at the anodic potential, allowing adsorption peaks for adenosine to be observed. Hydrogen peroxide is not adsorption controlled (Figure S-1 in the Supporting Information) and does not have any of the extra peaks. ATP has less of a secondary peak and fewer of the extra peaks than adenosine, likely because less adsorbs due to its charge. The extra peak on the downward scan is also observed for adenine oxidation but is much smaller in current (Figure S-3 in the Supporting Information). Adenine is the nucleobase of adenosine and does not contain the ribose unit. Because the extra peak is observed for adenosine, adenine, and ATP but not hydrogen peroxide, it must be due to an adsorption product of the nucleobase.

Sawhorse Waveform Optimization. The sawhorse waveform plateau potential and time were optimized for sufficient sensitivity and stability of adenosine. Figure 3 shows the effect of plateau potential (Figure 3A) and plateau time (Figure 3B). A range of plateau voltages were tested, from 1.25 to 1.45 V (n = 4). Very little current was detected at 1.25 and 1.30 V, which are below the oxidation potential for adenosine.[17] A noticeable jump in sensitivity was observed at 1.35 V and the current for this potential was significantly higher than both 1.25 and 1.30 V (one-way ANOVA with Bonferroni post-test, $p < 0.01$ and $p < 0.05$ respectively, $n = 4$). Slightly higher currents were detected at 1.40 and 1.45 V; however, the amount of current was not significantly different than 1.35 V (one-way ANOVA with Bonferroni post-test, $p > 0.05$). Thus, 1.35 V was chosen as the optimal plateau potential because it provided significantly more current than lower voltages but was further away from the potential for water hydrolysis. In addition, background currents were more stable at lower potentials.

Increasing the plateau time increases the current detected for 1 μM adenosine at a 1.35 V plateau potential (Figure 3B, one-way ANOVA main effect of time, $p = 0.0006$, $n = 4$). The shortest plateau time (0.5 ms) resulted in the least amount of current detected. Both 1.0 and 1.5 ms plateau times were significantly higher than 0.5 ms (one-way ANOVA with Bonferroni post-test, $p < 0.01$ and $p < 0.001$, respectively); however, 1.0 ms was not significantly different than 1.5 ms ($p > 0.05$). The background current was less stable for 1.5 ms so 1.0 ms was chosen as optimal. This plateau time is longer than that optimized for dopamine by Keithley et al.;[13] however, the purpose here was to allow more time for oxidation so a longer hold time was necessary. The stability of the optimized waveform in prefrontal cortex slices was assessed by application of 25 μM adenosine every 30 min for 2 h (Figure S-4 in the Supporting Information), and on average, the current detected did not change in that time period.

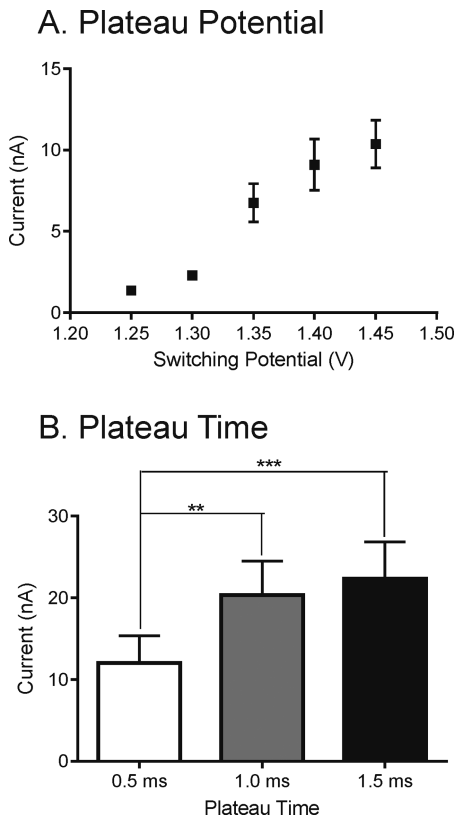

Figure 3. Optimization of the sawhorse waveform switching potential and plateau time. (A) A range of plateau voltage spanning from 1.25 to 1.45 V was tested. The plateau time is constant at 1.0 ms. A noticeable jump in current for 1 μM adenosine is seen at 1.35 V. Overall current was significantly dependent on switching potential (one-way ANOVA, $p = 0.0273$) and the current with 1.35 V was significantly higher than both 1.25 and 1.30 V (Bonferroni post test, $p < 0.01$ and $p < 0.05$, respectively, $n = 4$). Slightly higher currents were detected at 1.40 and 1.45 V; however, the amount of current was not significantly different than 1.35 V (one-way ANOVA with Bonferroni post test, $p > 0.05$, $n = 4$). (B) Three plateau times were tested: 0.5, 1.0, and 1.5 ms for 5 μM adenosine. The plateau voltage was held constant at 1.35 V. Overall, current was significantly dependent on plateau time (one-way ANOVA, $p < 0.001$). Both 1.0 and 1.5 ms plateau times were significantly higher than 0.5 ms (Bonferroni post-test, $p < 0.01$ and $p < 0.001$, respectively, $n = 4$); however, 1.0 ms was not significantly different than 1.5 ms ($p > 0.05$).

The sawhorse waveform produced significantly more current for adenosine than the triangle waveform at 1.30 and 1.35 V switching potentials (Figure 4, unpaired t test $p < 0.01$ and $p < 0.001$, respectively). With a 1.35 V upper potential, 1.3 ± 0.3 nA/μM adenosine was detected with the triangle waveform (n = 6), whereas 6.8 ± 1.1 nA/μM adenosine was detected with the sawhorse ($n = 6$); therefore, the sawhorse waveform offers a significant, 5-fold increase in current over the triangle waveform at 1.35 V (unpaired t test, $p < 0.001$, $n = 6$). The currents for 1.40 and 1.45 V were not significantly different between the sawhorse and triangle waveform (unpaired t test $p > 0.05$). The

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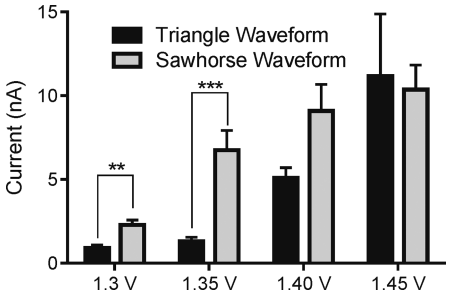

Figure 4. Comparison of current at both the triangle and sawhorse waveform at various switching potentials. The plot shows average current for each switching potential tested for both the triangle (black) and sawhorse (gray) waveform for 1 μM adenosine. The sawhorse waveform produced significantly more current for adenosine than the triangle waveform at 1.30 and 1.35 V switching potential (unpaired t test $p < 0.01$ and $p < 0.001$, respectively, $n = 6$).The currents for 1.40 and 1.45 V were not significantly different between the sawhorse and triangle waveform (unpaired t test $p > 0.05$, $n = 6$).

limit of detection (LOD) for the triangle waveform is 34 ± 10 nM with a switching potential of 1.35 V and is 21 ± 3 nM with 1.45 V,[18] whereas the LOD is 12 ± 4 nM at the sawhorse waveform with a 1.35 V switching potential ($n = 6$). The LOD of the sawhorse waveform is significantly different than the triangle waveform with a 1.35 V switching potential (unpaired t test, $p < 0.05$) but not significantly different than the triangle waveform with a 1.45 V switching potential (unpaired t test, $p > 0.05$). The sawhorse waveform offers more sensitivity at lower potentials than the triangle waveform.

Analyte Differentiation Using Principal Component Analysis. Hydrogen peroxide fluctuations *in vivo* have been measured[29] and because the CV for H_2O_2 is similar to adenosine, the ability to distinguish between them would be beneficial. Carbon-fiber microelectrodes are more sensitive to adenosine than hydrogen peroxide (6 nA/μM vs 1.5 nA/μM, respectively) but being able to distinguish CVs would increase confidence that hydrogen peroxide interferences could be ruled out during adenosine monitoring *in vivo*. Because the analytes have different shapes for CVs with the sawhorse waveform, principal component analysis (PCA) was used to predict concentrations of analytes in mixtures for both the sawhorse and triangle waveform.

Principal component analysis has been used in the past for discriminating between dopamine and pH changes.[37,38] PCA was also used to predict dopamine concentrations in the presence of basic pH shifts, ascorbic acid, and dihydroxyphenylacetic acid (DOPAC).[37] With PCA, a training set is created spanning the physiologically relevant concentrations of the analytes. For adenosine, ATP, and dopamine, the training set was 0.2 μM, 0.5 μM, 1 μM, and 5 μM. The hydrogen peroxide training set contained 10 μM, 20 μM, 30 μM, and 50 μM to match physiological concentrations and because our electrodes are not as sensitive to hydrogen peroxide. From the training set, eigenvalues are calculated; the largest eigenvalues correspond to the principal components with the highest variance and thus best correlate to the data.[39] A residual Q-score from the training set is used to reject data that does not significantly match the principal components. A training set was compiled for each analyte individually with each waveform and then mixtures of analytes were tested and PCA used to predict the concentration of each analyte in the mixture.

Mixtures of adenosine with hydrogen peroxide, ATP, or dopamine were analyzed using both the triangle and sawhorse waveform. Tables 1 and 2 show adenosine predictions in the presence of hydrogen peroxide, ATP, or dopamine for the triangle and sawhorse waveform, respectively. The first column of values is from a mixture of 5 μM adenosine and 10 μM hydrogen peroxide. For the triangle waveform, PCA underestimated the adenosine and overestimated the hydrogen peroxide concentration in the mixture (Table 1). Table S-1 in the Supporting Information gives statistical data using t tests that show the predicted adenosine and H_2O_2 concentrations are significantly different from the actual values. Small amounts of ATP and dopamine were also predicted, when none were present. In comparison, for the sawhorse waveform, PCA predicted concentrations of adenosine and hydrogen peroxide that were much closer to the actual concentration and negligible amounts of ATP and dopamine were predicted (Table 2, column 1, Table S-1 in the Supporting Information for statistics). The second column of values gives predicted concentrations from a mixture of 5 μM adenosine and 1 μM ATP. Again, with the triangle waveform, the adenosine concentration was underestimated and the ATP concentration overestimated (Table 1). A large portion of the adenosine and ATP mixture was attributed to hydrogen peroxide, which was not present. However, for the sawhorse waveform, the predicted values were closer to the actual values of adenosine and ATP and very little hydrogen peroxide was predicted (Table 2). Lastly, the third column of values in Tables 1 and 2 is predicted concentrations for a mixture of 5 μM adenosine and 1 μM dopamine. As with the other mixtures, the principal component analysis was much better at predicting the concentrations at the sawhorse waveform and did not predict high amounts of hydrogen peroxide or ATP, which were not present. A mixture of three analytes was also tested at the sawhorse waveform: 5 μM adenosine, 10 μM H_2O_2, and 1 μM

Table 1. Predicted Values for Triangle Waveform[a]

	H_2O_2 (10 μM)	ATP (1 μM)	DA (1 μM)
AD (5 μM)	3.2 ± 0.2	3.7 ± 0.2	2.8 ± 0.2
H_2O_2	19.3 ± 0.7	5.3 ± 0.3	5.8 ± 0.2
ATP	0.8 ± 0.4	1.5 ± 0.2	1.1 ± 0.2
DA	1.6 ± 0.8	0.3 ± 0.1	1.9 ± 0.1

[a]Table represents average predicted values of mixtures for the triangle waveform. Column 1 shows average predictions of the mixture of 5 μM adenosine (AD) and 10 μM hydrogen peroxide (H_2O_2). Column 2 is the mixture of 5 μM adenosine and 1 μM ATP. Column 3 is the mixture of 5 μM adenosine and 1 μM dopamine (DA). Values are mean ± SEM ($n = 4$).

Table 2. Predicted Values for Sawhorse Waveform[a]

	H_2O_2 (10 μM)	ATP (1 μM)	DA (1 μM)
AD (5 μM)	4.4 ± 0.3	4.5 ± 0.2	4.9 ± 0.3
H_2O_2	11 ± 1.0	0.5 ± 0.3	0.3 ± 0.3
ATP	0.6 ± 0.2	1.2 ± 0.1	0.3 ± 0.2
DA	0.00	0.05 ± 0.03	1.3 ± 0.3

[a]Table represents average predicted values of mixtures for the sawhorse waveform. Column 1 shows average predictions of the mixture of 5 μM adenosine (AD) and 10 μM hydrogen peroxide (H_2O_2). Column 2 is the mixture of 5 μM adenosine and 1 μM ATP. Column 3 is the mixture of 5 μM adenosine and 1 μM dopamine (DA). Values are mean ± SEM ($n = 4$).

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ATP. On average 3.7 ± 0.3 μM adenosine, 9.4 ± 0.7 μM hydrogen peroxide, and 1.2 ± 0.2 μM ATP were predicted, which were similar to the values predicted for mixtures of two components. Hydrogen peroxide and ATP predictions were not significantly different than actual values; however, the adenosine prediction was different than the actual value (unpaired t test, p = 0.0038, n = 5), as the model underpredicted its concentration. Thus, it is harder to distinguish mixtures of three components than two components.

All the values predicted for the triangle waveform (except for the ATP prediction in the adenosine/ATP mixture) were significantly different than the actual concentrations (Table S-1 in the Supporting Information, unpaired t test, $p < 0.001$). However, for the sawhorse waveform, predicted values were not significantly different than the actual values for the two component mixtures (Table S-1 in the Supporting Information). Thus, the sawhorse waveform in conjunction with principal components analysis is good for discriminating hydrogen peroxide from adenosine and predictions with PCA are more accurate than using the triangle waveform. Adenosine and ATP are the hardest to distinguish with either waveform; however, the sawhorse waveform was able to predict concentrations of ATP and adenosine in a mixture more accurately. While pharmacology would also be useful *in vivo* to help discriminate ATP and adenosine, this method is the best electrochemical method currently available for determining both in a mixture.

Mechanically Stimulated Adenosine Release Is Predicted As Adenosine in Brain Slices. Previously, mechanically stimulated adenosine release in the prefrontal cortex was characterized.[40] Lowering the electrode 50 μm in the brain slice caused adenosine release that was confirmed to be only adenosine by using pharmacological tests and enzyme sensors specific for adenosine and ATP. Mechanically stimulated adenosine release was also detected immediately after lowering a glass pipet of similar size near the working electrode. Here, we measured mechanically stimulated adenosine release in the prefrontal cortex with the sawhorse waveform. An in slice training set was generated by applying adenosine, ATP, and hydrogen peroxide in the slice after mechanical stimulation data had been collected (Figure 5A). The analytes were applied in a range of amounts to achieve different local concentrations at the electrode, just like the *in vitro* training set, and the concentrations at the electrode were calculated based on a precalibration factor. The calculated adenosine concentration based on the precalibration was compared to the adenosine concentration predicted by PCA in order to verify the accuracy of the prediction. Mechanically stimulated adenosine release (Figure 5B) has the same features as the exogenously applied adenosine (Figure 5A). Mechanically stimulated release does have an extra negative peak at the beginning of the current versus time plot, likely due to tissue disturbance from moving a glass pipet in tissue which could cause an ionic change (the negative peak was previously observed with the triangle waveform in past studies).[40] The extra peak may also be an unidentified molecule released during mechanical perturbation. Nevertheless, the PCA predictions were as expected, as the predicted signal was predominantly adenosine with negligible amounts of ATP and hydrogen peroxide (Figure 5C). The actual concentration and predicted concentrations of adenosine were not significantly different from one another (unpaired t test, $p > 0.05$, n = 8). This experiment proved that the sawhorse waveform could be used in tissue to predict adenosine concentrations.

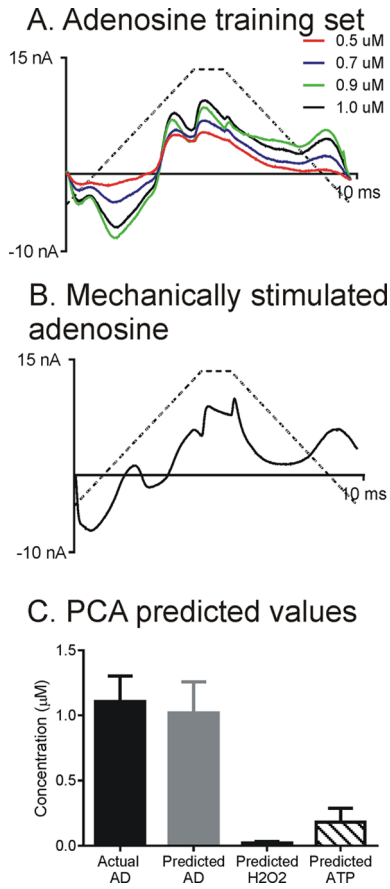

Figure 5. Mechanically evoked adenosine using the sawhorse waveform. The medial prefrontal cortex of a rat brain slice was mechanically stimulated by a glass pipet lowered approximately 30 μm away from the carbon-fiber microelectrode. After mechanical stimulation, an in slice training set was collected for adenosine, hydrogen peroxide, and ATP via exogenous application near the electrode. (A) An example adenosine training set in a slice. (B) An example of a mechanically evoked adenosine CV in a slice. (C) A comparison of the predicted values using PCA for the sawhorse waveform compared to the actual value if the release was all adenosine (black bar). The actual concentration and predicted concentrations of adenosine were not significantly different from one another (unpaired t test, $p > 0.05$, n = 8). Negligible amounts of hydrogen peroxide and ATP were predicted.

The shape of the adenosine CVs at the sawhorse waveform in slices changed slightly from *in vitro*, likely due to the differences in the tissue environment versus buffer. These differences dictate that an *in situ* training set must be used, as has been used for all previous PCA work.[41] For example, PCA has been used to identify adenosine transients *in vivo*, but the training set was large *in vivo* transients detected with the triangle waveform.[27] For dopamine, stimulated release *in vivo* was used as the training set to predict the concentration of spontaneous dopamine transients.[37,42] Here, we applied analytes to generate an in slice calibration set and the shapes of the mechanically stimulated adenosine release match well with the in slice calibration set.

In addition to predicting mechanically stimulated adenosine release in the prefrontal cortex, PCA with the sawhorse waveform was used to analyze mixtures of adenosine and ATP

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before and after administering an ATP metabolism inhibitor, POM-1. ATP can break down to adenosine on the millisecond time frame;[43] thus, predicting mixtures of adenosine and ATP in a slice, not in the presence of a drug, could be convoluted. In this experiment, an in slice training set for adenosine and ATP was generated and then a mixture of 25 μM adenosine and 25 μM ATP (1:1 ratio) was applied. Next, the brain slice was bathed in 100 μM POM-1 in aCSF for 30 min and the mixture of adenosine and ATP was applied again. PCA was used to predict the concentrations of adenosine and ATP that reached the electrode before and after POM-1 and the ratio of ATP to adenosine in the mixture was compared. The predicted ratio of ATP/adenosine before POM-1 was 1.3 ± 0.4 and after POM-1 it was 5.3 ± 1.3 (paired t test, $p = 0.0426$, $n = 5$). Since POM-1 blocks ATP from breaking down to adenosine, an increase of the ATP/adenosine ratio was expected. On average, after POM-1 the predicted adenosine decreased to 69 ± 18% of initial adenosine and ATP increased to 286 ± 66%. This experiment shows that the sawhorse waveform is useful for assessing ratios of adenosine and ATP in tissue.

Advantages of Modified Waveforms versus Modified Electrodes. Electrode modifications with polymers and/or carbon nanotubes have been used extensively in the past to increase sensitivity and specificity but they require extra fabrication time and cost of materials.[1,18,44,45] Nafion-CNT modified electrodes have enhanced sensitivity and selectivity for adenosine over ATP but the shape of the voltammograms were not different for ATP and adenosine and the sensitivity for hydrogen peroxide was never characterized.[18] Carbon nanotube yarns have been recently characterized for adenosine and hydrogen peroxide detection. While adenosine also has a secondary peak with those materials, discrimination of the two analytes was not tested.[46] Enzyme sensors for adenosine or ATP can be used to accurately measure each compound separately, but a single sensor cannot discriminate between mixtures and endogenous H_2O_2 can be an interfering agent because the enzymes ultimately produce and electrochemically detect H_2O_2.[47] Overall, the sawhorse waveform provides analyte discrimination of adenosine, ATP, and hydrogen peroxide and could be used to further enhance the detection of other neurochemicals *in vivo*.

CONCLUSIONS

In conclusion, a new waveform was developed for adenosine, hydrogen peroxide and ATP detection. The sawhorse waveform was first implemented to increase electrode stability at high scan rates as holding at the plateau oxidized and renewed the electrode surface.[13] We used the regular 400 V/s scan rate but focused on maximizing current for analytes that oxidize at the plateau potential. The sawhorse waveform allowed a lower switching potential than the traditional triangle waveform to be used and produced lower limits of detection. With the sawhorse waveform, adenosine has a different shape at the plateau potential and extra peaks due to adsorbed products; thus, it can be distinguished from dopamine, ATP, and hydrogen peroxide. PCA was used to predict concentrations in mixtures and in slices, confirming that the sawhorse waveform is better for discriminating adenosine in a mixture. Mechanically stimulated adenosine in prefrontal cortex slices was accurately predicted as adenosine using the sawhorse waveform. Overall, the sawhorse waveform is highly beneficial for analyte differentiation and could be used in the future *in vivo* to provide better selectivity at lower potentials.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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