Facilitation of Quantal Release Induced by a D1-like Receptor on Bovine Chromaffin Cells†

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ABSTRACT: Dopaminergic receptors are found on bovine adrenal chromaffin cells and have been implicated in the facilitation of an inward calcium current [Artalejo et al., (1990) *Nature 348*, 239−242] that could enhance release. However, previous studies using incubations of long duration (minutes) with dopaminergic receptor antagonists have found instead an inhibition of catecholamine release. In this work we used brief (subsecond) chemical depolarizing stimuli to reexamine the role of dopaminergic receptors on exocytosis from bovine adrenal chromaffin cells. Responses to consecutive depolarizing stimuli were compared using amperometry to monitor vesicular release events and intracellular fura-2 to examine Ca^{2+} dynamics within individual cells. Restoration of intracellular Ca^{2+} levels to their initial values following exposure to 60 mM K^+ was found to be prolonged unless the exposure was brief (0.5 s) and the cells were maintained at 37 °C. However, with these optimum conditions, a second stimulation evoked more exocytotic events than the first. This effect was blocked by SCH-23390, a D1 antagonist, in a dose dependent fashion, but not by raclopride, a D2 antagonist. The D1 agonist, SKF-38393, enhanced the number of exocytotic events as did prior exposure of the cell to epinephrine. Taken together, the data indicate that released catecholamines can enhance their own release by interaction with a D1-like receptor on bovine adrenal chromaffin cells.

Exocytotic release of chemical messengers is a common mechanism for communication between biological cells. This process is tightly regulated, and several controlling mechanisms have been identified including the influx of Ca^{2+} from the extracellular space, the normal trigger for exocytosis, and the regulation of the number of vesicles in the readily releasable pool. At many cells that undergo exocytosis, autoreceptors play an important regulatory role. For example, at most neurons autoreceptor activation inhibits further release. For example, activation of serotonin somatodendritic autoreceptors inhibits subsequent synthesis and release (*1*). Autoreceptors on dopaminergic neurons are D2-like (the D2 receptor class is composed of the D_2, D_3, and D_4 dopaminergic receptors), and dopamine autoreceptor effects are absent in mice with a genetic deletion of the D_2 receptor (*2*). Similar autoreceptor control has been shown for norepinephrine (*3*). An exception to the general inhibitory nature of neuronal autoreceptors appears to be glutamatergic synapses in the entorhinal cortex of juvenile rats that contain NMDA autoreceptors that enhance release (*4*).

Nonneuronal cells that secrete by exocytosis also have autoreceptors. Aspinwall et al. demonstrated that insulin-stimulated pancreatic β-cells secrete insulin (*5*). Deletion of insulin receptors from pancreatic β-cells results in a dramatically lowered level of insulin secretion (*6*). Thus, in contrast to neurons, these autoreceptors appear to promote increased release. Inhibitory autoreceptor regulation has been reported at bovine chromaffin cells from the adrenal gland. Early investigations, prompted by the phylogenetic relationship between chromaffin cells and sympathetic neurons that are well established to have autoreceptors, determined that autoreceptors on bovine chromaffin cells had properties that pharmacologically resemble dopaminergic receptors (*7*, *8*), and their activation inhibited release. Subsequent research showed that D2 dopamine receptors are located on bovine chromaffin cells and their activation inhibited release (*9*). A D1-like receptor (the D1 receptor class is composed of the D_1 and D_5 dopaminergic receptors) has also been identified on bovine chromaffin cells (*10*). Activation of this receptor facilitates an inward Ca^{2+} current that could promote exocytosis (*10*). However, experimentally it was found that D1 receptor activation actually inhibited catecholamine secretion (*11*).

In this work we describe a reinvestigation of the action of the D1 receptor on exocytosis from bovine chromaffin cells. All of the prior secretion experiments that investigated autoreceptors on bovine chromaffin cells exposed them to secretagogues for multiple minutes. In contrast, the D1-mediated facilitation Ca^{2+} currents were measured on a subsecond time scale. Therefore, in this work we evaluated release from bovine chromaffin cells using similar subsecond stimuli. This was accomplished by first optimizing the temperature and timing for K^+-based depolarization of individual chromaffin cells, while monitoring in real time release via amperometry and internal Ca^{2+} with fura-2. With release evoked by brief stimuli at 37 °C we found clear

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evidence for facilitation of exocytotic release mediated by a D1-like receptor on the surface of bovine chromaffin cells.

EXPERIMENTAL PROCEDURES

Cultured Chromaffin Cells. Bovine chromaffin cells were prepared from adrenal glands obtained from a local abattoir as previously described (*12*). Chromaffin cells were obtained from the adrenal medulla via digestion with collagenase (Worthington Biochemical Corporation, Lakewood, NJ). Density gradient centrifugation using Renografin was used to obtain a culture solution epinephrine-enriched fraction of cells. Cells were plated at a density of 3×10^5 cells/35 mm diameter plate (Becton Dickinson, Franklin Lakes, NJ) containing a 25 mm glass coverslip (Carolina Biological, Burlington, NC). Cell culture plates were kept at 37 °C in a 5% CO_2-supplemented incubator (Fisher Scientific, Hampton, NH). Cells were used days 3−7 postplating.

Electrodes and Electrochemistry. Carbon-fiber disk microelectrodes were prepared from T-650 fibers as previously described (*13*). Carbon fibers were aspirated into glass capillaries. A pipet puller (Narishige, Long Island, NY) was used to seal the glass around the carbon fiber. The carbon fibers were cut at the glass seal, and the seal was reinforced with epoxy (15% *m*-phenylenediamine mixed with Epon 828 resin (Miller-Stephenson, Danbury, CT)) heated to 80 °C. The electrodes were cooled at room temperature for 12 h and cured at 100 °C for 12 h, followed by curing at 150 °C for 12 h. Before use, electrodes were beveled at 45° on a diamond polishing wheel (Sutter Instruments, Novato, CA) using impedance monitoring and soaked in isopropyl alcohol for a minimum of 20 min (*14*). Constant potential amperometry was used to monitor exocytotic events and was measured using an Axopatch 200B (Axon Instruments, Molecular Devices, Union City, CA). The microelectrode was held at 0.650 V vs an Ag/AgCl reference (Bioanalytical Systems, West Lafayette, IN); individual release events were detected as current spikes. The resulting signal was filtered at 5 kHz with a low-pass Bessel filter and collected at 10 kHz. Amperometric recordings were then filtered at 400 Hz using a Gaussian filter.

Release Measurements. Culture plates containing cells in buffer (in mM) 145 NaCl, 5 KCl, 1 $MgCl_2$, 11.2 glucose, 10 HEPES,[1] and 2 $CaCl_2$) were placed on the stage of a Zeiss 35 inverted microscope (Zeiss, Thornwood, NY). The position of the carbon-fiber microelectrode and stimulating pipet was controlled with piezoelectric micromanipulators (Burleigh Instruments, Exfo, Plano, TX). Exocytosis was evoked with 60 mM K^+ delivered by pressure ejection from the stimulating pipets that were constructed with a horizontal puller (Sutter Instruments, Novato, CA). The diameter of the pipet tip was adjusted to 10 μm using a microforge (Narishige, Long Island, NY). Ejection of the high K^+ solution was accomplished with a Picospritzer set at 7 psi (General Valve Corporation, Parker Hannifin, Fairfield, NJ) using either a 0.5 or 2 s bolus. The stage holding the cell plate was either kept at room temperature, 25 °C, or heated with a water bath (Fisher Scientific, Hampton, NH) to 37 °C.

Ca^{2+} Measurements. Ca^{2+}-imaging employed the fluorescent dye, fura-2, and an imaging system (Empix, Inc., Mississauga, ON, Canada) attached to a microscope (Nikon, Lewisville, TX). An initial fura-2 solution was diluted with 40 μL of DMSO and 10 μL of 10% pluronic. This fura-2 solution was diluted to 1 μM in experimental buffer containing 0.10% BSA (Molecular Probes, Invitrogen, Carlsbad, CA). Cells were incubated in this solution for 20 min at 25 °C, followed by a rinse for 20 min at 25 °C in the experimental buffer. Ca^{2+}-imaging experiments were conducted either simultaneously with amperometry or alone. Intracellular fura-2 bound to Ca^{2+} was excited at 340 nm while free fura-2 was excited at 380 nm. Emission was monitored at 510 nm using a digital CCD camera and software (Empix Imaging, Inc, Mississauga, ON, Canada). The ratio of the emission with 340 nm excitation and 380 nm excitation was determined. The ratiometric measurements were then converted to $[Ca^{2+}]_i$ as previously described (*15*, *16*). The area under the Ca^{2+} response curve was determined with MiniAnalysis (Synaptosoft, Decatur, GA). The intracellular level directly before a stimulation was taken as the baseline.

Autoreceptor Experiments. Experiments were conducted at 37 °C using an S2/S1 protocol. The number of exocytotic events following an initial delivery (S1) of 60 mM K^+ for 0.5 s was compared to a second delivery of 60 mM K^+ (S2) 10 or 30 s later. To evaluate D1 receptors, the D1 antagonist, SCH-23390 (1 μM, 10 μM, 100 μM Sigma-Aldrich, St. Louis, MO), was present in the buffer. To evaluate D2 receptors, the D2 antagonist, raclopride (1 μM, 10 μM, 100 μM Sigma-Aldrich, St. Louis, MO), was present in the buffer. To evaluate D1 receptors via a D1 agonist, SKF-38393 was introduced to the buffer. Paired 0.5 s boluses spaced 30 s apart were used to deliver the 60 mM K^+ secretagogue to cells. At 10 s after the initial stimulus, 100 μM SKF-38393 (Sigma-Aldrich, St. Louis, MO), a D1 agonist, was delivered to the buffer and remained present during the second stimulus.

To evaluate the endogenous ligand, a 3 s bolus of 50 μM epinephrine was applied to the cell 22 s after S1 and S2 was applied 5 s later. As a control, the experiment was repeated at separate cells with ejection of experimental buffer substituted for epinephrine.

Data Analysis and Statistics. Spike analysis was performed using MiniAnalysis. For selection as spikes, the amplitude had to exceed the root-mean-square current noise by a factor of 5. Student's *t* test and one-way ANOVA were used to determine significance among data sets. A value of $p < 0.05$ was taken to indicate a significant difference.

RESULTS

Vesicular Release and Ca^{2+} Dynamics Evoked by 0.5 s and 2 s Stimuli at 25 °C. At 25 °C, a 0.5 s pressure ejection of 60 mM K^+ onto a chromaffin cell was sufficient to cause Ca^{2+} influx into the cell, measured by fura-2 fluorescence, and this was accompanied by exocytotic spikes measured by amperometry (example traces in left panel of Figure 1). Intracellular $[Ca^{2+}]$ reached a maximum after the 60 mM K^+ ejection was terminated and then slowly returned to

[1] Abbreviations: SCH-23390, 1*H*-3-benzazepin-7-ol, 8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-, (5*R*); SKF-38393, 1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol; GnRH, gonadotropin-releasing hormone; HEPES, 1-[4-(2-hydroxyethyl)-1-piperazinyl]ethane-2-sulfonic acid; NMDA, *N*-methyl-D-aspartic acid; OT, oxytocin.

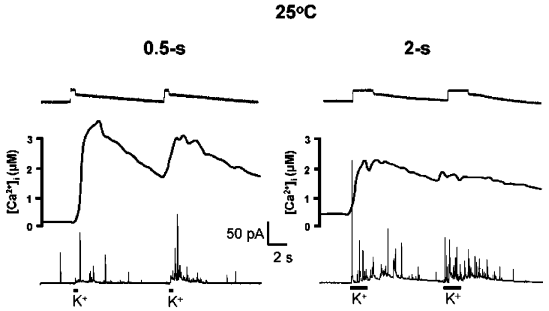

FIGURE 1: Comparison of 0.5 s and 2 s exposures to 60 mM K^+ at 25 °C. Stimulations were paired with a 10 s interstimulus interval. Uppermost traces show bolus of 10 μM dopamine applied to electrode to show ejection profile. Middle traces show $[Ca^{2+}]_i$ collected with fura-2; bottom traces show amperometric traces of release. Right and left panels show 0.5 s and 2 s stimulations, respectively.

Table 1: Spike Number Ratios, S2/S1, at 25 and 37 °C[a]

	25 °C		37 °C	
interstimulus interval (s)	0.5 s stimulus	2 s stimulus	0.5 s stimulus	2 s stimulus
10	1.1 ± 0.3	1.0 ± 0.1	2.0 ± 0.1	1.2 ± 0.2
20	1.0 ± 0.2	0.69 ± 0.10	1.6 ± 0.4	1.7 ± 0.2
30	1.8 ± 0.3	1.2 ± 0.1	1.6 ± 0.1	1.3 ± 0.2
40	1.3 ± 0.2	0.76 ± 0.10	1.7 ± 0.4	1.5 ± 0.2

[a] Spike number ratios, S2/S1, are shown at 25 °C for 0.5 s at 10, 20, 30, and 40 s intervals (n = 5, 7, 13, 12, respectively) and 2 s stimuli for 10, 20, 30, and 40 s intervals (n = 5, 22, 23, 13, respectively). Spike number ratios are also shown at 37 °C for 0.5 s at 10, 20, 30, and 40 s intervals (n = 10, 9, 16, 10, respectively) and 2 s stimuli for 10, 20, 30, and 40 s intervals (n = 13, 11, 17, 10, respectively).

baseline. The decay of the $[Ca^{2+}]_i$ was sufficiently slow that it failed to reach the original level within 10 s of the initial K^+ exposure. A second, 0.5 s pressure ejection of 60 mM K^+, 10 s after the first, caused a smaller increase in $[Ca^{2+}]_i$ that returned to baseline. Again, exocytosis was observed during the time $[Ca^{2+}]_i$ was elevated.

To quantify the effects of consecutive 0.5 s stimuli on the occurrence of exocytotic events at 25 °C, we examined the ratio of the number of spikes evoked on a second K^+ exposure (S2) to those evoked on the first (S1). If preceding stimuli do not affect subsequent exocytotic events, this spike number ratio should be unity. The ratios were examined with different intervals between the stimuli to allow increasing recovery times (Table 1). With a 10 s or 20 s interval between 0.5 s, 60 mM K^+ exposures, the spike number ratios were approximately unity, but they exceeded unity with 30 or 40 s between stimuli. The ratio of the areas under the $[Ca^{2+}]_i$ curves was also calculated as an S2/S1 ratio. With a 10 s interval between stimuli, the S2/S1 $[Ca^{2+}]_i$ area ratio was 0.45 ± 0.06, but increased to a value between 0.7 and 0.8 with longer intervals between stimuli (Table 2). The average $[Ca^{2+}]_i$ area ratio measured across the four intervals was 0.71 ± 0.04 and was significantly depressed from unity (p < 0.05).

Similar experiments were done at 25 °C with 2 s pressure ejections of 60 mM K^+. With the longer stimulus, the $[Ca^{2+}]_i$ reached a maximum during the stimulus, evoking exocytosis, but again was slow to return to prestimulus values (see right panel of Figure 1). A second exposure 10 s later did not evoke much of a change in $[Ca^{2+}]_i$, but did evoke further

Table 2: $[Ca^{2+}]_i$ Area Ratios, S2/S1, at 25 and 37 °C[a]

	25 °C		37 °C	
interstimulus interval (s)	0.5 s stimulus	2 s stimulus	0.5 s stimulus	2 s stimulus
10	0.45 ± 0.06	0.18 ± 0.05	1.2 ± 0.1	0.82 ± 0.04
20	0.88 ± 0.03	0.57 ± 0.10	1.2 ± 0.1	0.65 ± 0.06
30	0.79 ± 0.07	0.51 ± 0.05	1.1 ± 0.1	0.61 ± 0.04
40	0.71 ± 0.08	0.47 ± 0.05	0.87 ± 0.05	0.52 ± 0.06

[a] $[Ca^{2+}]_i$ area ratios, S2/S1, are shown at 25 °C for 0.5 s at 10, 20, 30, and 40 s intervals (n = 5, 7, 9, 12, respectively) and 2 s stimuli for 10, 20, 30, and 40 s intervals (n = 5, 13, 17, 14, respectively). $[Ca^{2+}]_i$ area ratios are also shown at 37 °C for 0.5 s at 10, 20, 30, and 40 s intervals (n = 6, 6, 6, 7, respectively) and 2 s stimuli for 10, 20, 30, and 40 s intervals (n = 5 for all intervals).

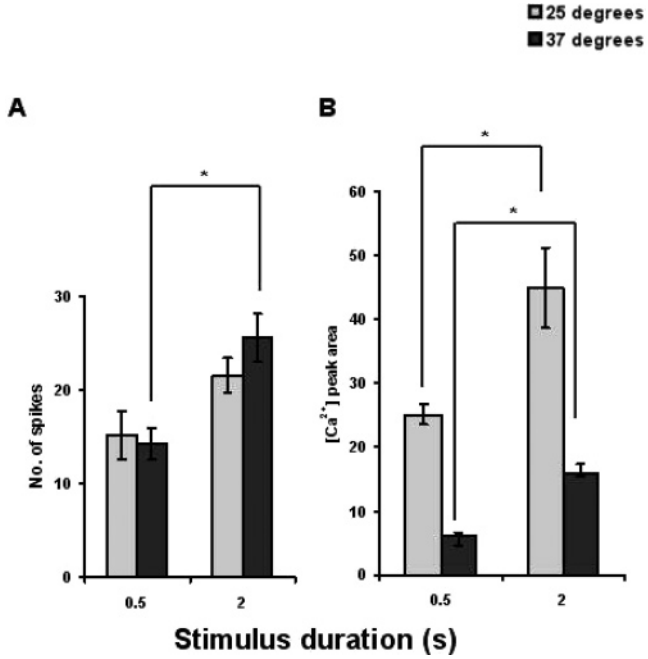

FIGURE 2: Effects of stimulus time and temperature on vesicular release and Ca^{2+} influx. (A) Number of spikes elicited using 0.5 s (n = 53) and 2 s stimuli (n = 42) at 25 °C was not statistically significant. There was a significant difference between the number of spikes elicited at the two stimuli at 37 °C (p < 0.05). Temperature did not show an effect on number of evoked spikes at a particular stimulus duration. (B) Ca^{2+} dynamics in response to temperature and stimulus conditions. Ca^{2+} area during evoked Ca^{2+} influx using 0.5 s (n = 5) and 2 s stimuli (n = 7) at 25 °C was statistically significant (p < 0.05); 0.5 s stimuli (n = 25) and 2 s stimuli (n = 20) at 37 °C also evoked average Ca^{2+} areas that were significantly different (p < 0.05). Ca^{2+} dynamics are more sensitive to temperature and stimulus duration.

exocytosis. Surprisingly, the number of spikes evoked on the first exposure with 2 s exposure to 60 mM K^+ (21.5 ± 1.8) was not significantly different from that evoked with a 0.5 s pulse (15.1 ± 2.5; $p \geq 0.05$; Figure 2A). However, the $[Ca^{2+}]_i$ area with the 2 s pressure ejection (44.95 ± 6.29 arbitrary units) was significantly greater than with a 0.5 s exposure (25.08 ± 1.54 arbitrary units; p < 0.05; Figure 2B).

The spike number ratios for consecutive stimuli using the 2 s stimulus were also found to be variable and near unity, but no consistent trend with interval between the stimuli was seen (Table 1). The $[Ca^{2+}]_i$ area ratio was very low with a 10 s interval between 2 s exposures to 60 mM K^+, and it increased for longer intervals (Table 2). The average

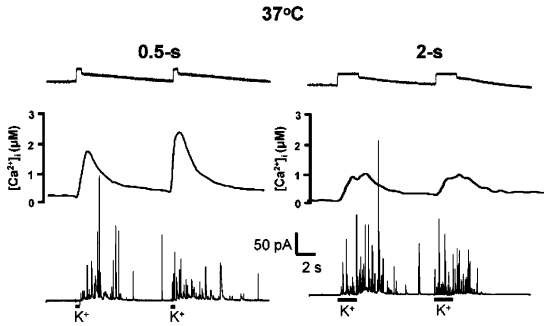

FIGURE 3: Comparison of 0.5 s and 2 s exposures to 60 mM K^+ at 37 °C. Stimulations were paired with a 10 s interstimulus interval. Uppermost traces show bolus of 10 μM dopamine applied to electrode to show ejection profile. Middle traces show $[Ca^{2+}]_i$ collected with fura-2; bottom traces show amperometric traces of release. Right and left panels show 0.5 s and 2 s stimulations, respectively.

measured over the four intervals was 0.43 ± 0.05, which was significantly lower than unity ($p < 0.05$), again indicating insufficient time for recovery before the second stimulus. Clearly, the 2 s stimulus at 25 °C sufficiently perturbs the mechanisms that regulate $[Ca^{2+}]_i$ such that the responses on the second exposure differ considerably from those on the first exposure.

Vesicular Release and Ca^{2+} Dynamics Evoked by 0.5 s and 2 s Stimuli at 37 °C. Identical experiments were done at 37 °C. At this temperature, the Ca^{2+} responses were more rapid (Figure 3). With a 0.5 s delivery of 60 mM K^+, $[Ca^{2+}]_i$ was maximal soon after the stimulus, and returned to near baseline within 10 s, and multiple exocytotic spikes were observed. With a 10 s interval between stimuli, the spike number ratio approached 2, and it was above 1.5 with longer intervals between stimuli (Table 1). These S2/S1 spike number ratios evoked during 0.5 s exposures were significantly higher than unity ($p < 0.05$) indicating facilitation of release on the second stimulation at 37 °C. The $[Ca^{2+}]_i$ area ratios were above unity with a 10 s interval between stimuli, and remained high until the stimuli were separated by 40 s (Table 2).

At 37 °C, a 2 s stimulus of 60 mM K^+ caused Ca^{2+} influx that was maximal during the ejection, and which decayed to baseline within 10 s. In addition, there were significantly more spikes evoked with a 2 s bolus (25.5 ± 2.5) than with a 0.5 s bolus (14.2 ± 1.7; $p < 0.05$, Figure 2).

The spike number ratio was 1.2 with 10 s between 2 s ejections of 60 mM K^+ at 37 °C (Table 1). With longer intervals between stimuli, the spike number ratio hovered between 1.3 and 1.7; these spike number ratios were significantly higher than unity, also indicating the presence of facilitation ($p < 0.05$). With all time intervals, the average $[Ca^{2+}]_i$ area ratio with 2 s pressure ejections was less than unity (Table 2). The average ratio over the 10, 20, 30, and 40 s intervals was 0.65 ± 0.01, which is significantly different from unity ($p < 0.05$). Thus, even at 37 °C, 2 s exposures to 60 mM K^+ perturbed the cell sufficiently that $[Ca^{2+}]_i$ could not achieve the initial evoked value on subsequent stimuli, at least with the interstimulus times investigated. Note that while there was little change in the number of spikes evoked at the two different temperatures, the $[Ca^{2+}]_i$ area ratios were significantly smaller at 37 °C with both ejection durations (Figure 2). This is because

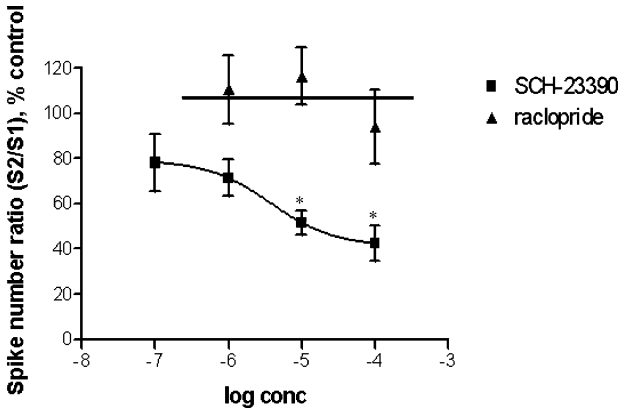

FIGURE 4: Dose response curve of D1 and D2 antagonists. Data was obtained using 0.5 s K^+ stimuli at 37 °C with 10 s intervals. To investigate the presence of a D2-receptor mediated effect on facilitation, raclopride, a D2 antagonist, was incubated with cells at 1, 10, and 100 μM (n = 5, 10, 7, respectively). The S2/S1 responses were compared to control values measured at the same cell. Various concentrations of SCH-23390 (0.1, 1, 10, 100 μM; n = 8, 7, 7, 8, respectively) were also used to examine its effect on facilitation. Spike number ratios obtained at 10 and 100 μM SCH-23390 were significantly different from control values ($p < 0.05$).

$[Ca^{2+}]_i$ is more rapidly restored to low levels at the higher temperature.

Facilitation and Depression of Vesicular Release via the D1 Receptor. To investigate whether the elevated number of spikes seen following a second 0.5 s exposure to 60 mM K^+ at 37 °C was due to specific receptor interactions, we examined the effects of selective pharmacological agents. In the first experiment, the effect of different concentrations of the D1-receptor antagonist, SCH-23390, on the spike number ratio was examined with 0.5 s K^+ pressure ejections that were 10 s apart. The average S2/S1 spike number ratio without drug for the 10 s interval yielded a mean of 2.2 ± 0.2. This facilitation decreased progressively with increasing concentrations of SCH-23390 (Figure 4). Facilitation was significantly decreased related to experiments without drug ($p < 0.05$) in the presence of SCH-23390 at concentrations of 10 and 100 μM.

To test whether D2 receptors were participating in this effect on release, the D2 antagonist, raclopride, was used in an analogous study at doses of 1 μM, 10 μM, and 100 μM (Figure 4). For this set of experiments, the average spike number ratio, S2/S1, without drug was 2.2 ± 0.3 with 0.5 s exposures 10 s apart. Changes in spike number ratios were not observed at any of the concentrations tested.

Next, the effect of a D1-receptor agonist was evaluated. In these experiments, the K^+ stimuli were 30 s apart because, under control conditions (Table 1), facilitation was not as pronounced as with the shorter intervals. SKF-38393 (100 μM), a D1-receptor agonist, was introduced into the buffer 10 s after the first 0.5 s K^+ pressure ejection. The spike number ratio was 2.5 ± 0.4 following the agonist compared to 1.2 ± 0.2 ($p < 0.05$) in the absence of the agonist (Figure 5). The facilitation caused by SKF-38393 was blocked when 10 μM of the D1-receptor antagonist, SCH-23390, was included in the buffer ($p < 0.05$; Figure 5).

Epinephrine-Induced Facilitation. To test whether the physiological ligand that is secreted by chromaffin cells

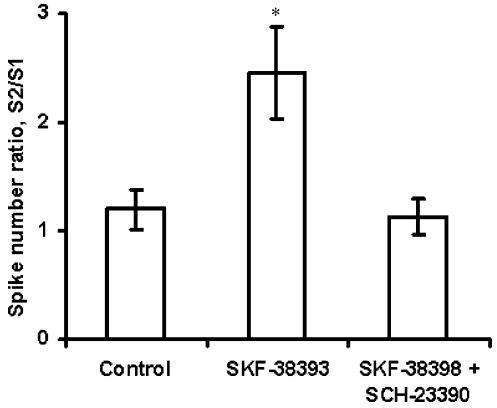

FIGURE 5: D1 receptor-mediated facilitation. Experiments were conducted with a 30 s interstimulus interval. Spike number ratios showed recovery in control cells ($n = 9$) and facilitation in cells incubated with 100 μM SKF-38393, a D1 agonist ($n = 9$). Coincubation of 100 μM SKF-38393 and 10 μM SCH-23390 showed inhibition of the facilitation effect ($n = 5$). Incubation with SKF-38393 alone was significantly different from control value and coincubation of SKF-38393 and SCH-23390 ($p < 0.05$).

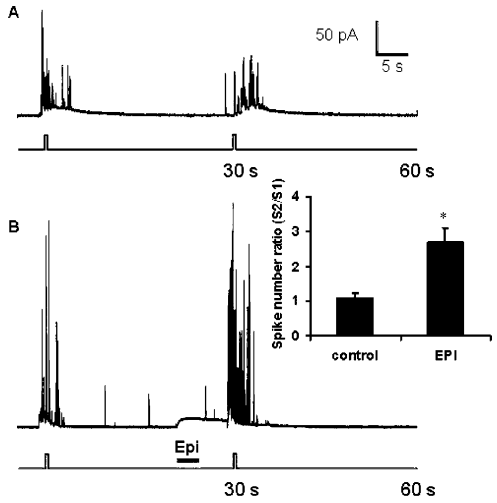

FIGURE 6: Epinephrine-induced facilitation. Experiments were conducted with a 30 s interstimulus interval. (A) Control trace where experimental buffer was applied to the cell for 3 s records recovery of release upon the second stimulus, S2. (B) Release was recorded with 3 s application of 50 μM epinephrine. S2 in these cells showed a greater number of elicited spikes. Note the baseline change due to detection of the pressure ejection of epinephrine. Inset shows average S2/S1 values for control cells and cells that received brief applications of epinephrine ($n = 12$). The values were shown to be significantly different ($p < 0.05$).

would elicit facilitation, the experiment was repeated with a transient application of 50 μM epinephrine. Epinephrine was applied as a 3 s bolus 22 s after the first 0.5 s bolus of K^+; 5 s after completion of the epinephrine application, the second 0.5 s bolus of K^+ was applied to the cell. Epinephrine evoked facilitation in release as was seen with the D1 agonist, SKF-38393 (Figure 6). Transient application of 50 μM epinephrine to chromaffin cells yielded a spike number ratio of 2.7 $\pm$ 0.4 (Figure 6, inset) which was 265 $\pm$ 44% of the control ratio that was obtained by pressure ejecting experimental buffer instead of epinephrine (1.1 $\pm$ 0.1; $p < 0.05$; Figure 6).

To test whether the effect of epinephrine was D1 receptor-mediated, two consecutive S2/S1 protocols were used. In the first, epinephrine was introduced 5 s before S2. Then,

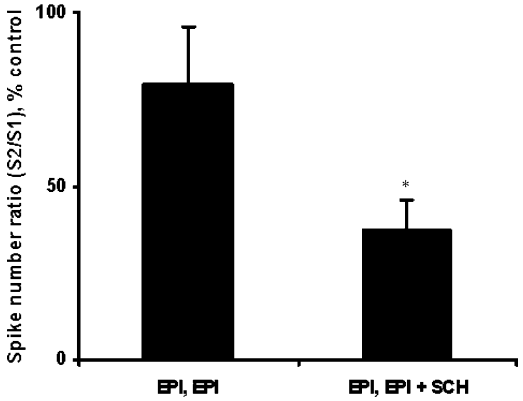

FIGURE 7: Epinephrine-induced facilitation is D1-modulated. Experiments were conducted with 30 s interstimulus interval. To investigate if the epinephrine-induced facilitation was mediated by a D1-like receptor, SCH-23390 was used to attempt to block the effect. A pair of S2/S1 stimuli were evaluated at each cell with 50 μM epinephrine applied during S2; after 5 min, a second set of identical paired stimuli was used with transient application with ($n = 5$) or without 10 μM SCH-23390 ($n = 7$). Values are shown as the percent of the initial S2/S1 spike number ratios.

the experiment was repeated 5 min later, with or without a D1-receptor antagonist. The second epinephrine application in the absence of drug showed facilitation, but its S2/S1 spike number ratio was 79 $\pm$ 17% of the first (Figure 7). This may indicate desensitization that is occurring at the receptor. However, in the presence of SCH-23390 (10 μM) the facilitation effect was blocked. The ratio of S2/S1 spike number ratios was 37.4 $\pm$ 8.7%, which was significantly different than the response in the absence of drug ($p < 0.05$; Figure 7).

DISCUSSION

The results described here demonstrate that there is an autoreceptor on bovine chromaffin cells that facilitates release of epinephrine. Previous work has demonstrated the presence of dopaminergic D1 (*8*, *10*) and D2 (*9*) receptors on bovine chromaffin cells. Consistent with those findings, chromaffin cells have been shown to express RNA for the D_4 and D_5 dopaminergic receptors (*11*). Additionally, it has been shown that D1 receptor activation (presumably through activation of the D_5 receptor) causes facilitation of Ca^{2+} currents (*10*). Since it was postulated that the Ca^{2+} influx linked to the D1 receptor was sufficient to evoke further release (*17*), we tested for this effect. Clear evidence for facilitation of release was found at physiological temperature and with subsecond exposure to small amounts of K^+. This facilitation was blocked by a D1 receptor antagonist in a dose-dependent manner. The facilitation could be mimicked by a D1 agonist as well as the endogenous secreted species, epinephrine. Thus, we have established a functional role for the previously identified D1 receptor on bovine chromaffin cells.

Crucial to our observation of facilitated autoreceptor-dependent release was the optimization of the stimulus conditions. Rapid application of secretagogues was enabled by the fabrication of ejection pipets that permit delivery of a relatively sharp concentration profile even on a 0.5 s time scale (*18*). The pipet tips have a diameter of 10 μm or less, which minimizes leakage of the secretagogue from the tip. The paired stimuli approach that employed a 0.5 s or 2 s exposure to 60 mM K^+ was sufficient to promote release of

multiple vesicles with each exposure. Since the electrode samples release from approximately 6% of the cell surface, each 0.5 s exposure can be estimated from the data in Figure 2 to release approximately 250 vesicles. Because one estimate of the readily releasable pool is approximately 175 (*19*) of the total 10 000 vesicles in the cell (*20*), the majority of our release is believed to come from this compartment.

The spike number ratio was always unity or greater with the 0.5 s stimuli. In contrast, with 2 s stimuli, the spike number ratio was often lower than unity at 25 °C, suggesting that the cell (or the vesicle pools and their associated release mechanisms) on the second stimulus was recovering from the first stimulus (*21*). Consistent with prior work, the number of spikes initially evoked by the same stimulus duration was similar at both temperatures (*22*, *23*). The strongest evidence that the cell is dramatically perturbed with the 2 s stimuli comes from the $[Ca^{2+}]_i$ area ratios. Only with 0.5 s stimuli at 37 °C were $[Ca^{2+}]$ area ratios near unity achieved. The cells were able to recover more effectively at 37 °C as the $[Ca^{2+}]_i$ was restored to near resting levels even within a 10 s interstimulus interval. Intracellular Ca^{2+} levels are primarily maintained by uptake into mitochondria (*24*, *25*), an energy dependent process that is more effective at physiological temperature (*26*). Thus, we used the 0.5 s stimulus at 37 °C to investigate further receptor-mediated facilitation of release. With short (10 s) intervals between stimuli under these conditions, the $[Ca^{2+}]$ area ratios exceeded unity, although the ratios were not as great as the facilitated Ca^{2+} currents reported by Artalejo and co-workers (*27*). As well as reporting on cytoplasmic Ca^{2+}, fura-2 also buffers it and, because our measurements are of whole cell fluorescence, this average measure of Ca^{2+} is expected to be much lower than the enhanced Ca^{2+}currents measured with patch clamp technology (*28*).

To test the hypothesis that facilitation of exocytosis is mediated by interaction of a released substance with a D1 dopamine receptor (*17*), the conditions that showed facilitation were repeated in the presence of SCH-23390, a D1 antagonist. While this agent blocked facilitated release in a dose-dependent manner, facilitated release was unaffected by the D2 antagonist, raclopride. The doses of SCH-23390 needed to inhibit facilitation are quite high. Two possibilities may contribute to this finding. First, the binding of SCH-23390 to sites on chromaffin cell is considerably weaker than to other tissues that have D1 receptor sites (*29*). Because of this, we are probably more correct to refer to this receptor as a "D1-like receptor." Second, the assay used involves the competition between released epinephrine and the antagonist. Further evidence that a D1-like receptor is involved in facilitation was obtained with the D1 agonist, SKF-38393. This agent induced exocytotic facilitation in a 30 s paired pulse interval, a paradigm that normally shows limited facilitation.

Consistent with the concept that facilitation is autoreceptor-mediated, epinephrine induced robust facilitation of the number of vesicular release events at a modest concentration compared to the surface concentration upon its release (*30*). Desensitization appears to occur because the epinephrine-induced facilitation was somewhat diminished upon repeating the application (Figure 7). However, the facilitation induced by epinephrine appears receptor-mediated because it was inhibited by the D1-receptor antagonist, SCH-23390. Previously, it was proposed that intracellular or plasma dopamine was the primary species responsible for activating dopaminergic receptors on bovine chromaffin cells (*9*, *10*). This work shows that released epinephrine could be the catecholamine activating the D1-like receptor. Indeed, epinephrine is well-known to have a high affinity for the D1 receptor (IC_{50} = 68 nM) (*31*). Epinephrine is in abundance in this preparation since the majority of chromaffin cells package and release epinephrine; the ratio of epinephrine- to norepinephrine-containing cells is approximately 70%:30% (*32*). Moreover, the experiments performed in this work were conducted on the epinephrine-enriched fraction of cells obtained from the cell culture preparation. Therefore, the observed facilitation originates by binding of epinephrine to a functional D1-like autoreceptor.

Several earlier studies demonstrated a D2 receptor-mediated inhibition of catecholamine release (*7*−*9*). Furthermore, previous reports of D1 receptor-mediated secretion at chromaffin cells suggested a decrease in release (*11*). However, many of these were done at room temperature, a condition not explored here. Both D1 and D2 mediated effects were ascribed to inhibition of sodium uptake that would cause an attenuated membrane depolarization (*33*). We attribute the difference in responses obtained here to differences in the time scale of secretagogue application. Prior studies of catecholamine release from bovine chromaffin cells used incubation times of minutes. In this work each secretagogue exposure was transient and attempted to mimic physiological conditions of a brief burst of action potentials. D1 receptor activation may have predominantly short-term effects, as indicated by our results, while the effect of D2 receptor activation may become predominant over longer time periods.

The D1 receptor-mediated facilitation that occurs in chromaffin cells points to a positive feedback cycle that operates with paired stimuli that are spaced closely together. Previously, using a paired stimuli approach, an overfilling of the releasable pool of vesicles was noted at relatively short interstimulus intervals (approximately 2−10 s) in bovine chromaffin cells (*21*). The autoreceptor that we have functionally characterized in this work may be the origin of this apparent overfilling. By activating facilitating Ca^{2+} currents, more Ca^{2+} is available to stimulate greater release. Autoreceptor-mediated positive feedback occurs in other systems as well. In addition to the positive feedback observed with application of insulin to β-pancreatic cells (*5*), another positive feedback regulator is gonadotropin-releasing hormone (GnRH). GnRH potentiates the pulsatile secretion of GnRH neurons *in vitro* (*34*). GnRH agonists were also shown to potentiate the pulsatile neuronal response while GnRH antagonists had an inhibitory effect. Moreover, oxytocin (OT) and its agonist enhance bursting activity in OT neurons while the OT antagonist inhibits this same response (*35*). In the chromaffin cell, which is part of the "fight-or-flight" mechanism, a positive feedback system is desirable. In this way, the body is flooded with adrenaline in order to be prepared for high stress situations. Closely spaced stimuli would signal to these cells the urgent need for the rapid release of large amounts of catecholamine into the bloodstream and initiate the positive feedback cycle necessary for an immediate response to the presented threat or stress.

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