P2Y Purinoceptors Inhibit Exocytosis in Adrenal Chromaffin Cells via Modulation of Voltage-Operated Calcium Channels

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We have used combined membrane capacitance measurements (C_m) and voltage-clamp recordings to examine the mechanisms underlying modulation of stimulus–secretion coupling by a $G_{i/o}$-coupled purinoceptor (P2Y) in adrenal chromaffin cells. P2Y purinoceptors respond to extracellular ATP and are thought to provide an important inhibitory feedback regulation of catecholamine release from central and sympathetic neurons. Inhibition of neurosecretion by other $G_{i/o}$-protein-coupled receptors may occur by either inhibition of voltage-operated Ca^{2+} channels or modulation of the exocytotic machinery itself. In this study, we show that the P2Y purinoceptor agonist 2-methylthio ATP (2-MeSATP) significantly inhibits Ca^{2+} entry and changes in C_m evoked by single 200 msec depolarizations or a train of 20 msec depolarizations (2.5 Hz). We found that P2Y modulation of secretion declines during a train such that only ~50% of the modulatory effect remains at the end of a train. The inhibition of both Ca^{2+} entry and ΔC_m are also attenuated by large depolarizing prepulses and treatment with pertussis toxin. Inhibition of N-type, and to lesser extent P/Q-type, Ca^{2+} channels contribute to the modulation of exocytosis by 2-MeSATP. The Ca^{2+}-dependence of exocytosis triggered by either single pulses or trains of depolarizations was unaffected by 2-MeSATP. When Ca^{2+} channels were bypassed and exocytosis was evoked by flash photolysis of caged Ca^{2+}, the inhibitory effect of 2-MeSATP was not observed. Collectively, these data suggest that inhibition of exocytosis by $G_{i/o}$-coupled P2Y purinoceptors results from inhibition of Ca^{2+} channels and the Ca^{2+} signal controlling exocytosis rather than a direct effect on the secretory machinery.

Key words: P2Y purinoceptor; G-protein-coupled receptor; voltage-operated calcium channels; exocytosis; modulation; presynaptic inhibition; adrenal chromaffin cells

It is now well established that ATP can act as a fast excitatory neurotransmitter by activation of postsynaptic purinoceptors (Edwards et al., 1992; Evans et al., 1992) (for review, see Zimmermann, 1994, Gibb and Halliday, 1996). Recent studies have shown that ATP may also act as a neuromodulator. Activation of presynaptic P2X purinoceptors facilitates neurotransmission (Khakh and Henderson, 1998; Boehm, 1999) (for review, see MacDermott et al., 1999), whereas activation of presynaptic P2Y purinoceptors is thought to inhibit neurotransmission (Von Kügelgen et al., 1989; Boehm, 1999). The mechanism(s) underlying inhibition of neurotransmission by P2Y purinoceptors is unknown. Presynaptic inhibition of neurotransmitter release by other G-protein-coupled receptors (GPCRs), however, is generally thought to involve either changes in membrane excitability and Ca^{2+} signaling or a direct effect on some component of the release machinery (Hille, 1994; Wu and Saggau, 1997; Miller, 1998). Central to the Ca^{2+} hypothesis for presynaptic inhibition are the observations that neuronal, somatic voltage-operated Ca^{2+} channels are inhibited by $G_{i/o}$-proteins (Dolphin, 1998) and that pharmacologically similar channels control exocytosis (Dunlap et al., 1995). It is assumed that the modulatory processes that regulate Ca^{2+} channels in the soma also occur at release sites. The release machinery hypothesis, on the other hand, arises from the observation that activation of GPCRs inhibits exocytosis when Ca^{2+} channels are bypassed or blocked (Silinsky, 1986; ManSonHing et al., 1989; Shen and Surprenant, 1990; Lang et al., 1995). Whether modulation of the release machinery contributes significantly to GPCR-dependent inhibition of stimulus-evoked neurotransmitter release is still a matter of debate (Thompson et al., 1993; Miller, 1998).

The aim of this study was to determine the mechanisms underlying modulation of exocytosis by P2Y purinoceptors. The system we chose to study was the adrenal chromaffin cell because it is well established that these cells secrete catecholamines by Ca^{2+}-regulated exocytosis (Neher, 1998) and they express inhibitory P2Y purinoceptors that couple to neuronal Ca^{2+} channels (Diverse-Pierluissi et al., 1991; Gandia et al., 1993; Currie and Fox, 1996). Moreover, chromaffin cells co-store and release ATP along with catecholamine (Winkler and Westhead, 1980), and it is thought that, as in sympathetic neurons (Von Kügelgen et al., 1994), ATP may act as an autocrine regulator of secretion from these cells (Carabelli et al., 1998). We used combined voltage-clamp and membrane capacitance measurements (C_m) to study the effects of P2Y purinoceptor activation on stimulus–secretion coupling. Our results show that the selective P2Y agonist 2-methylthio ATP (2-MeSATP) inhibited both Ca^{2+} channels and ΔC_m via a voltage-dependent, pertussis toxin (PTX)-sensitive mechanism. The P2Y-mediated inhibition of exocytosis was not associated with a change in the Ca^{2+}-dependence of secretion. When Ca^{2+} channels were bypassed and exocytosis was stimulated by flash photolysis of nitrophenyl-EGTA (NP-EGTA), 2-MeSATP had no effect on ΔC_m. These data provide strong evidence in favor of the Ca^{2+} hypothesis for presynaptic

inhibition by $G_{i/o}$PCR and may also explain feedback inhibition of catecholamine release through P2Y purinoceptors.

MATERIALS AND METHODS

Chromaffin cell culture. Chromaffin cells were prepared by collagenase digestion of bovine adrenal glands according to the method of Trifaro and Lee (1981). Adrenal glands from 18- to 24-month-old cows were obtained from a local abattoir and were retrogradely perfused at 25 ml/min for 30 min at 37°C with the digestive enzymes 0.03% collagenase type 2 (Worthington Biochemical Corp., Lakewood, NJ) and 0.0013% DNase I (Boehringer Mannheim, Indianapolis, IN) added to a Locke's solution consisting of (in mM): 154.2 NaCl, 2.6 KCl, 2.2 K_2HPO_4, 0.85 KH_2PO_4, 10 glucose, and 5 HEPES, with 0.0005% Phenol Red (Life Technologies, Paisley, UK), pH adjusted to 7.2 with NaOH. After surgical removal of the cortex, the medulla was dissected, cut into small pieces, placed in a trypsinization flask with fresh enzyme solution, and stirred at slow speed for 30 min at 37°C. Cells were washed twice with Earle's Balanced Salt Solution (Life Technologies) and resuspended in DMEM (Life Technologies) supplemented with 44 mM $NaHCO_3$, 15 mM HEPES, 10% fetal calf serum (Life Technologies), 1% glutamine, 1% penicillin–streptomycin solution, 2.5 mg/ml gentamycin, 0.5 mg/ml 5′-fluorodeoxyuridine, and 0.01 mg/ml cytosine-β-δ-arabino-furanoside. Cells were plated on glass coverslips coated with matrigel (Becton Dickinson, Bedford, MA) at a density of ~800 cells/mm². Approximately 80% of the media was replaced 24 hr after plating, and cells were maintained for up to 7 d in a humidified atmosphere of 95% O_2–5% CO_2 at 37°C.

Electrophysiological recordings. A coverslip carrying chromaffin cells was placed in a microperfusion chamber on the stage of an inverted phase-contrast microscope (Diaphot 200; Nikon, Tokyo, Japan). Cells were continuously superfused with an external solution consisting of (in mM): 130 NaCl, 2 KCl, 1 $MgCl_2$, 5 $CaCl_2$, 10 glucose, and 10 HEPES, adjusted to pH 7.2 with NaOH, osmolarity of ~280 mOsm. In some experiments, cells were superfused with a external solution in which the NaCl was replaced with equimolar tetraethyl ammonium (TEA) chloride. Tetrodotoxin was omitted because it slows Na^+ channel-gating kinetics 10-fold, resulting in a contamination of the C_m trace (Horrigan and Bookman, 1994). Drugs were prepared as stock solutions in double-distilled water, except where stated, and then diluted at least 1000-fold into external solution. Special care was taken to superfuse cells at a high rate (~ 3 ml/min) throughout the experiment and to select well isolated single cells for recording to avoid compounding effects of endogenously released modulators (Carabelli et al., 1998).

Ionic currents were recorded in perforated patch-clamp configuration using borosilicate glass electrodes coated with Sylgard 184 (Dow Corning, Midland, MI) and fire polished on a microforge to a resistance of 1–2 MΩ. Electrodes were filled with an internal solution consisting of (in mM): 145 Cs-glutamate (Calbiochem, Nottingham, UK), 10 HEPES, 9.5 NaCl, and 0.3 BAPTA (Molecular Probes, Eugene, OR), adjusted to pH 7.2 with CsOH (ICN Biomedicals, Aurora, OH), osmolarity ~280 mOsm. Gramicidin D (Sigma, Poole, UK) at a final concentration of 9.7 µg/ml (stock solution in DMSO 1080 µg/ml) was used for perforation. Flash photolysis experiments were performed as described by Parsons et al. (1996) using the whole-cell patch-clamp configuration and electrodes filled with an internal solution consisting of (in mM): 110 Cs-glutamate, 40 HEPES, 10 NP-EGTA (Molecular Probes), 7 $CaCl_2$, and 0.3 fura-2 pentapotassium salt (Calbiochem), adjusted to pH 7.2 with CsOH.

For both whole-cell and perforated patch recordings, series resistance was <12 MΩ and compensated (typically >70%) electronically using a patch-clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA). Voltage protocol generation and data acquisition were performed using custom data acquisition software (kindly provided by Dr. A. P. Fox, University of Chicago, Chicago, IL) running on a Pentium computer equipped with a Digidata 1200 acquisition board (Axon Instruments). Current traces were low-pass filtered at 5 kHz using the four-pole Bessel filter supplied with the amplifier and digitized at 10 kHz. Current traces were corrected off-line for linear leakage current (typically <10 pA, at −90 mV) using the P4 method. Chromaffin cells were voltage-clamped at −90 mV, and C_m was sampled with a resolution of 12 msec using a software-based phase-tracking method as described previously (Fidler and Fernandez, 1989; Seward et al., 1995). C_m measurements were interrupted during voltage-steps and then resumed 40 msec after the stimulus to exclude gating charge artifacts (Horrigan and Bookman, 1994; Chow et al., 1996). Data were stored on the computer hard drive and analyzed off-line using self-written analysis software (Axobasic;

Axon Instruments) and commercial software (Origin; Microcal, Northampton, MA). All experiments were performed at ambient temperature (21–25°C).

Photolysis of caged calcium and $[Ca^{2+}]_i$ measurements. Flashes of UV light were derived from a pulsed arc lamp (TILL Photonics, Planegg, Germany) coupled to the epi-illumination port of an Axiovert 100 inverted microscope equipped with a 40× oil immersion objective with a numerical aperture of 1.3 (Zeiss, Jena, Germany). UV flashes were restricted to a 240 µm spot using the objective, and the cell under investigation was placed in the center of this region. Fluorescence measurements and UV flashes were restricted to the area immediately surrounding the cell using rectangular field stops. To measure $[Ca^{2+}]_i$, the internal solution contained fura-2 (for composition, see above), and the cell was alternately illuminated at 340 and 380 nm using a monochromator (TILL Photonics) controlled by the C_m data acquisition software. Emission >430 nm was collected with a photomultiplier tube (TILL Photonics) and sampled at the rate of C_m measurements, approximately every 12 msec. Data were stored on a personal computer, and ratios of 340/380 nm were calculated off-line (Axobasic-written software). Calibration of fura-2 was performed by the method of Grynkiewicz et al. (1985). R_{min} and R_{max} were obtained by permeabilizing chromaffin cells with 10 µM ionomycin or 3 µM digitonin in the presence of 10 mM EGTA or 20 mM Ca^{2+}, respectively. In control experiments ($n = 4$) in which cells were loaded with Ca^{2+}-free NP-EGTA internal solution, flashes failed to produce either a rise in fura-2 ratio at 340/380 nm or C_m.

In some perforated patch experiments, cells were loaded with the Ca^{2+} indicator by addition of 5 µM fura-2 AM (Molecular Probes) to DMEM medium and incubating for 25 min at 37°C. Cells were then washed with fresh DMEM and incubated an additional 15 min at 37°C. The K_d for fura-2 alters according to the surrounding milieu (Grynkiewicz et al., 1985). Because the intracellular environment is unknown in the perforated patch configuration, coupled with difficulty in determining R_{min}, fura-2 measurements were not calibrated in these experiments and ratios are given in Results.

Data analysis. ΔC_m was measured relative to a 100 fF calibration signal, which was routinely switched in and out of the circuit during the course of a recording. The ΔC_m triggered by a single voltage pulse was calculated off-line. "Immediate" ΔC_m occurring during a voltage pulse was measured by averaging four C_m points immediately before the voltage pulse and subtracting this value from the average of the first four C_m points acquired immediately after the voltage pulse. "Slow" ΔC_m observed as drifts in C_m after membrane repolarization were measured by taking the average of the first four C_m points acquired immediately after the voltage pulse and subtracting them from the average of four C_m points taken at the peak of the C_m rise. Slow ΔC_m that exceeded twice the resting C_m noise (10–20 fF) were considered significant. Unless otherwise indicated, the total ΔC_m resulting from a single depolarization was taken as the sum of the immediate response and slow response. The total ΔC_m evoked by a train of depolarizations was calculated as the sum of immediate ΔC_m for all the pulses in the train plus any drifts in C_m occurring between pulses. The latter was calculated by taking the average of the first four C_m points acquired after a pulse and subtracting them from the average of four C_m points taken before the next voltage pulse.

Ca^{2+} entry was determined by integration of Ca^{2+} currents (I_{Ca}), and the left limit was set ~3 msec into the voltage pulse, to exclude the major portion of the contaminating Na^+ current. Before integration, currents were leak-subtracted using the P4 method. Statistical significance was determined using either paired or independent Student's *t* test, as appropriate. All data are expressed as the mean ± SEM. Data reported are taken from 67 cells from 20 separate cultures.

RESULTS

Previous whole-cell patch-clamp studies with Ba^{2+} as the divalent cation have shown that ATP inhibits I_{Ca} in adrenal chromaffin cells (Diverse-Pierluissi et al., 1991; Gandia et al., 1993). For our studies on exocytosis, we used the perforated patch configuration to avoid secretory rundown (Engisch and Nowycky, 1996; Seward and Nowycky, 1996), and we used Ca^{2+} as the charge carrier to obtain efficient secretion (Seward et al., 1996). In non-neuronal cells and the chromaffin-like PC12 cell line, P2Y purinoceptors are commonly found to be G_q-coupled and reported to increase rather than decrease $[Ca^{2+}]_i$ (for review, see Boeynaems et al., 1998). Therefore, in our first series of experiments, we examined
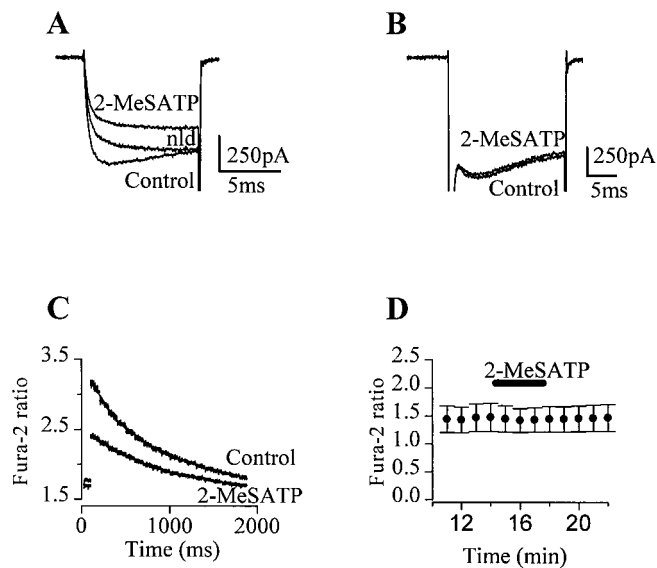
Figure 1. P2Y receptor activation inhibited I_{Ca} in a voltage-dependent, PTX-sensitive manner. *A, B,* Superimposed current traces during a 20 msec depolarization to +20 mV from a holding potential of −90 mV in the absence (*Control*) or presence of 100 nM 2-MeSATP. *A,* I_{Ca} recorded after equimolar replacement of NaCl in the superfusing solution with TEA-Cl to remove contaminating Na^+ currents. Under control conditions, 2-MeSATP reduced the maximal current amplitude and slowed the activation kinetics. The effect on kinetics can be seen by normalizing the current in the presence of 2-MeSATP to the control trace (*nld*). *B,* Treatment with PTX (250 ng/ml, 24 hr) occluded the inhibitory effect of 2-MeSATP on I_{Ca}. The transient current visible during the first 3 msec of the depolarization is a Na^+ current, which has been cut off for illustration purposes only. *C, D,* Effect of 2-MeSATP on $[Ca^{2+}]_i$ measured in cells loaded with the Ca^{2+} indicator fura-2 AM (see Materials and Methods). Data shown are the ratios of emitted fluorescence (510 nm) for excitation at 340 and 380 nm. *C,* Measurement of cell-averaged $[Ca^{2+}]_i$ recorded after a 30 msec depolarization to +20 mV before (*Control*) and in the presence of 2-MeSATP. *D,* Superfusion of 2-MeSATP (100 nM) did not induce any changes in the resting $[Ca^{2+}]_i$ recorded at −90 mV immediately before a voltage step (data shown is the mean ± SEM for *n* = 4).

the effects of P2Y purinoceptor activation on Ca^{2+} channels and $[Ca^{2+}]_i$ in isolated chromaffin cells under our recording conditions. Superfusion of 2-MeSATP, an ATP analog with preference for metabotropic purinoceptors, caused a reversible inhibition of I_{Ca} evoked by step depolarizations to +20 mV from a holding potential of −90 mV (Fig. 1*A*). The IC_{50} for 2-MeSATP inhibition of I_{Ca} was ~3 nM (A. D. Powell and E. P. Seward, unpublished observations). At a maximally effective concentration (100 nM), 2-MeSATP inhibited the Ca^{2+} entry integrated over 17 msec by 51 ± 3% (*n* = 5) and slowed current activation kinetics (Fig. 1*A*). Similar effects of 2-MeSATP were observed on I_{Ca} recorded using the whole-cell recording configuration in which 0.3 mM BAPTA was used to buffer $[Ca^{2+}]_i$ (47 ± 2% inhibition; *n* = 4). The I_{Ca} inhibition was voltage-dependent because application of a 20 msec prepulse to +120 mV reduced the inhibition of the integrated Ca^{2+} entry to 14 ± 4% (*n* = 3). Treatment of chromaffin cells with PTX (250 ng/ml, 24 hr) reduced the effect of 2-MeSATP (100 nM) on integrated Ca^{2+} entry to 3.5 ± 0.9% (*n* = 4) (Fig. 1*B*). In cells loaded with fura-2 AM to monitor $[Ca^{2+}]_i$ under perforated patch recording conditions, superfusion with 2-MeSATP did not produce any significant change in basal $[Ca^{2+}]_i$ (mean ratio at 340/380 nm excitation before agonist was 1.48 ± 0.24 and in the presence of 2-MeSATP was 1.44 ±

0.24; *n* = 4) (Fig. 1*D*), suggesting that there are no functional Ca^{2+}-mobilizing G_q-coupled P2Y purinoceptors or P2X purinoceptors. However, in these same cells, 2-MeSATP did inhibit peak stimulus-evoked $[Ca^{2+}]_i$ increases by 47 ± 6% (*n* = 4) (Fig. 1*C*). Together, these data indicate that activation of purinoceptors in bovine adrenal chromaffin cells leads to inhibition of Ca^{2+} entry through Ca^{2+} channels and that this effect is mediated by a PTX-sensitive $G_{i/o}$-protein.

P2Y purinoceptor activation inhibits ΔC_m evoked by single long depolarizations

Exocytosis in chromaffin cells and neurons is tightly regulated by Ca^{2+}, with Ca^{2+} entry through Ca^{2+} channels providing the trigger for secretory vesicle fusion (Seward and Nowycky, 1996; Neher, 1998). Thus, we predicted that inhibition of Ca^{2+} channels by P2Y purinoceptors in chromaffin cells would be accompanied by a decrease in exocytosis. To measure exocytosis, we combined voltage-clamp recording of Ca^{2+} channels with C_m measurements to monitor vesicle fusion. Exocytosis was evoked by single long (200 msec) depolarizations to +20 mV from a holding potential of −90 mV once every 3–5 min. Figure 2, *A* and *C*, shows the time course and results of a typical experiment. Empirically, we found that this stimulus protocol produced reproducible stimulus-evoked C_m increases without the activity-dependent changes in exocytotic efficiency (Fig. 2*E*) reported previously (Engisch et al., 1997; Smith, 1999). Before agonist application, the mean stimulus-evoked integrated Ca^{2+} entry over 197 msec was 342 ± 34 × 10^6 ions (*n* = 15). The resulting increases in C_m could be resolved into one or two kinetically distinct phases, depending on the cell. In all cells, Ca^{2+} entry was associated with an immediate increase in ΔC_m with a mean secretory rate of 1430 ± 190 fF/sec (*n* = 15) (Fig. 2*D*). In 6 of these 15 cells, the immediate ΔC_m was followed by a smaller, slow C_m increase that had a mean rate of 204 ± 56 fF/sec (Fig. 2D_2). We found no correlation between the peak amplitude of the I_{Ca} or integrated Ca^{2+} entry and the presence or absence of slow C_m increases.

Superfusion with 100 nM 2-MeSATP produced a reversible inhibition of stimulus-evoked exocytosis (Fig. 2*C,D*). The inhibition of the immediate and slow ΔC_m were, however, significantly different (*p* = 0.02) (Fig. 2*F*). 2-MeSATP produced a mean inhibition of immediate ΔC_m corresponding to 31 ± 5% (*n* = 15). The inhibition of Ca^{2+} entry integrated over 197 msec was 22 ± 3%. In cells with pronounced slow exocytosis, 2-MeSATP inhibited the slow ΔC_m by 52 ± 6% (*n* = 6). The larger inhibition of ΔC_m compared with Ca^{2+} entry is consistent with the nonlinear Ca^{2+}-dependence of exocytosis we (see Fig. 5*B*) and others have measured in chromaffin cells stimulated with long depolarizations (Engisch and Nowycky, 1996).

The inhibitory effects of 10 nM 2-MeSATP on Ca^{2+} entry and ΔC_m were less than those produced by 100 nM, and they were reduced in the presence of the purinoceptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (100 μM) from 16 ± 3 and 25 ± 7% to 8 ± 4 and 11 ± 2%, respectively (*n* = 3).

2-MeSATP inhibition of exocytosis involves N- and P/Q-type Ca^{2+} channels

Release of neurotransmitter is often associated with specific subtypes of Ca^{2+} channels (Dunlap et al., 1995). In chromaffin cells,
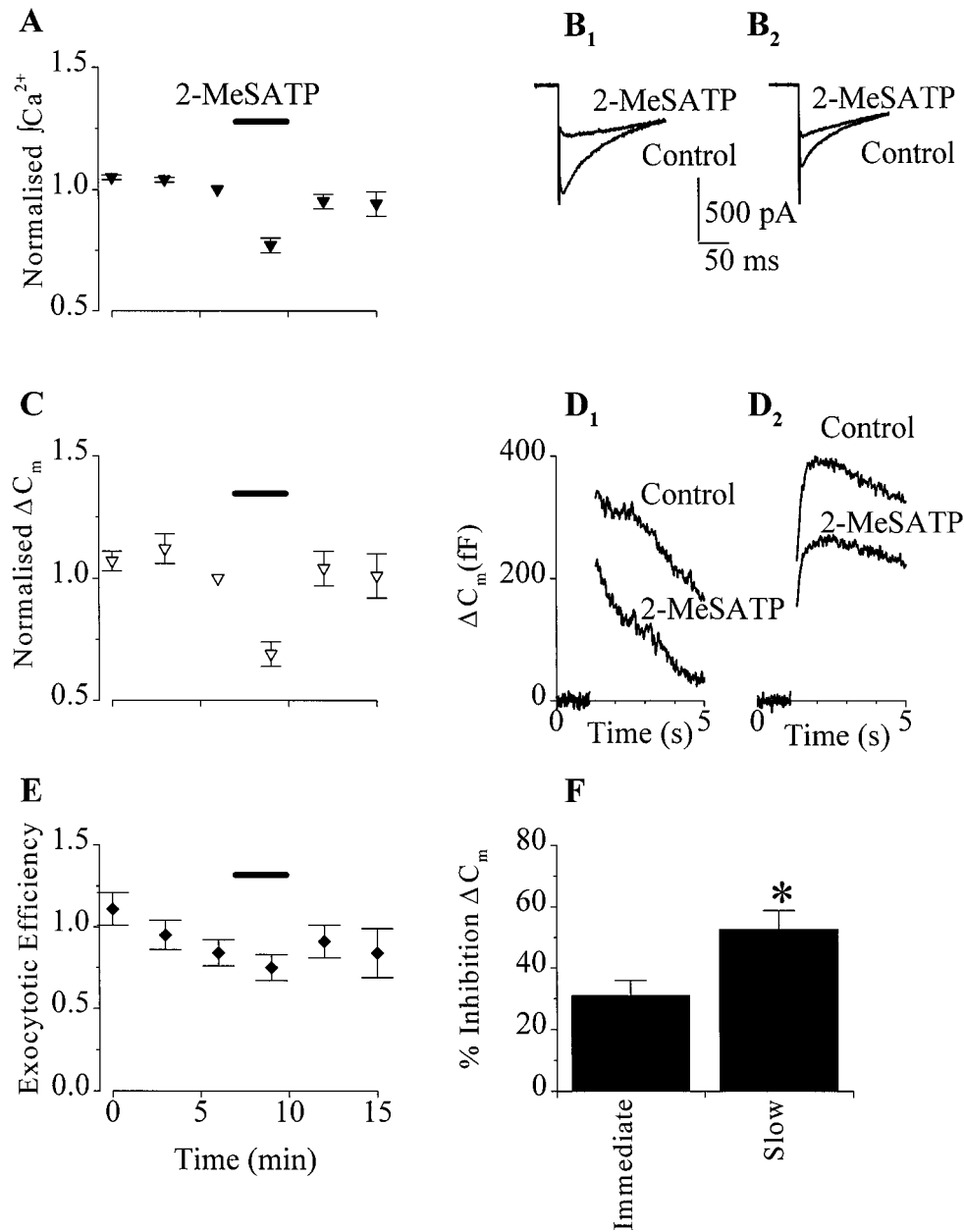
Figure 2. P2Y purinoceptor activation inhibited membrane capacitance (C_m) changes evoked by 200 msec depolarizations. *A, C, E,* Diary plots of the effect of 100 nM 2-MeSATP on normalized Ca^{2+} entry as measured by integrating I_{Ca} (*A*), ΔC_m (*C*), and exocytotic efficiency ($\Delta C_m/$ Ca^{2+} entry) (*E*) (data plotted are the mean ± SEM for $n = 15$). Both integrated Ca^{2+} entry and ΔC_m were normalized to the responses recorded immediately before 2-MeSATP application (*horizontal bar*). *B,* Superimposed currents recorded before (*Control*) and during superfusion with 2-MeSATP from two different chromaffin cells (B_1 and B_2). Currents were evoked by a 200 msec depolarization to +20 mV from −90 mV. *D,* ΔC_m recorded in response to currents shown in *B*. Gaps in the C_m traces represent when a voltage step was applied. Two kinetically distinct phases of C_m change were observed; in D_1, the response consisted solely of an immediate change in C_m and, in D_2, an additional slow component of exocytosis that appears as a drift up in C_m after the voltage step. 2-MeSATP inhibited both I_{Ca} (B_1 and B_2) and the corresponding immediate and slow ΔC_m (D_1 and D_2). *F,* Summary of the effect of 2-MeSATP on the immediate ($n = 15$) and slow ($n = 6$) ΔC_m. $* p < 0.02$ indicates significant difference between the effect of 2-MeSATP on immediate and slow ΔC_m.

the role of pharmacologically distinct Ca^{2+} channels in exocytosis depends on stimulus conditions and development (Artalejo et al., 1994; Engisch and Nowycky, 1996; Lomax et al., 1997). We examined the role of different subtypes of Ca^{2+} channels in mediating the inhibitory effects of 2-MeSATP on exocytosis. Application of ω-conotoxin GVIA (ω-CgTx GVIA) (1 μM) to block N-type Ca^{2+} channels inhibited Ca^{2+} entry by 29 ± 5% and inhibited ΔC_m evoked by single 200 msec depolarizations by 34 ± 5% ($n = 8$) (Fig. 3B_1). Blockade of P/Q-type Ca^{2+} channels with ω-agatoxin IVA (ω-Aga IVA) (300 nM) inhibited Ca^{2+} entry by 52 ± 9% and inhibited ΔC_m by 73 ± 9% ($n = 4$) (Fig. 3*A,B_1*). Combined application of ω-CgTx GVIA and ω-Aga IVA inhibited Ca^{2+} entry by 96 ± 1% and inhibited ΔC_m by 94 ± 2% ($n = 6$) (Fig. 3*A*). No significant contributions to stimulus-evoked exocytosis from a dihydropyridine-sensitive "facilitation" channel were observed, presumably because our studies were performed on adult animals and Ca^{2+}-dependent processes were not per-

turbed by using barium as the charge carrier. We conclude therefore that, under our recording conditions, Ca^{2+} entry through N- and P/Q-type channels triggers exocytosis. Furthermore, as observed with 2-MeSATP, the inhibition of secretion produced by either toxin was proportional to the inhibition of Ca^{2+} entry and did not alter the Ca^{2+}-dependence of exocytosis.

To investigate the contribution of each subtype of Ca^{2+} channel to the modulation of secretion by 2-MeSATP, we applied ω-CgTx GVIA (1 μM) or ω-Aga IVA (300 nM) alone to isolate P/Q- or N-type Ca^{2+} channels, respectively. 2-MeSATP (100 nM) inhibited Ca^{2+} entry and ΔC_m evoked by N-type Ca^{2+} channels by 46 ± 6 and 44 ± 7% ($n = 5$) (Fig. 3*A,B_2*), whereas Ca^{2+} entry and ΔC_m evoked by P/Q-type Ca^{2+} channels was inhibited by 18 ± 9 and 16 ± 5% ($n = 4$), respectively. The inhibitory effects of 2-MeSATP on Ca^{2+} entry and ΔC_m involving N-type channels was significantly greater than that involving P/Q-type channels ($p < 0.05$) (Fig. 3B_2).
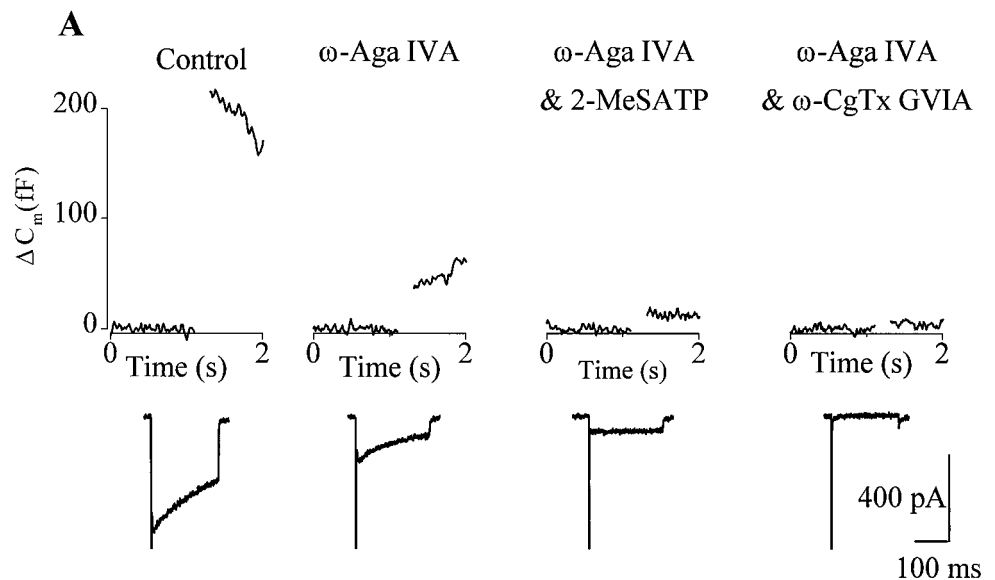
Figure 3. N- and P/Q-type Ca^{2+} channels mediated the inhibition of exocytosis by 2-MeSATP. *A,* Data shown are from a single cell and illustrate the effect of ω-Aga IVA (300 nM), 2-MeSATP (100 nM), and ω-CgTx GVIA (1 μM) on both the C_m changes (*top*) and the associated I_{Ca} (*bottom*) recorded in response to 200 msec depolarization to +20 mV from a holding potential of −90 mV. The voltage step is indicated by the gap in C_m trace. Both C_m changes and I_{Ca} were inhibited by ω-Aga IVA (*A, second panel*). The remaining C_m change and I_{Ca} were inhibited by 2-MeSATP (*A, third panel*) and could be completely blocked by combined addition of ω-Aga IVA and ω-CgTx GVIA (*A, fourth panel*). B_1, Summary of effects of the Ca^{2+} channel toxins ω-CgTx GVIA (*n* = 8) and ω-Aga IVA (*n* = 4) on integrated Ca^{2+} entry (*open bars*) and ΔC_m (*filled bars*). B_2, Summary of the effect of 2-MeSATP on integrated Ca^{2+} entry (*open bars*) and ΔC_m (*filled bars*) evoked by isolated Ca^{2+} channel subtypes (N-type, *n* = 5; P/Q-type, *n* = 4). ω-CgTx GVIA was used to isolate P/Q-type Ca^{2+} channels, and ω-Aga IVA was used to isolate N-type Ca^{2+} channels. Similar results were obtained irrespective of the order in which the toxins were applied.
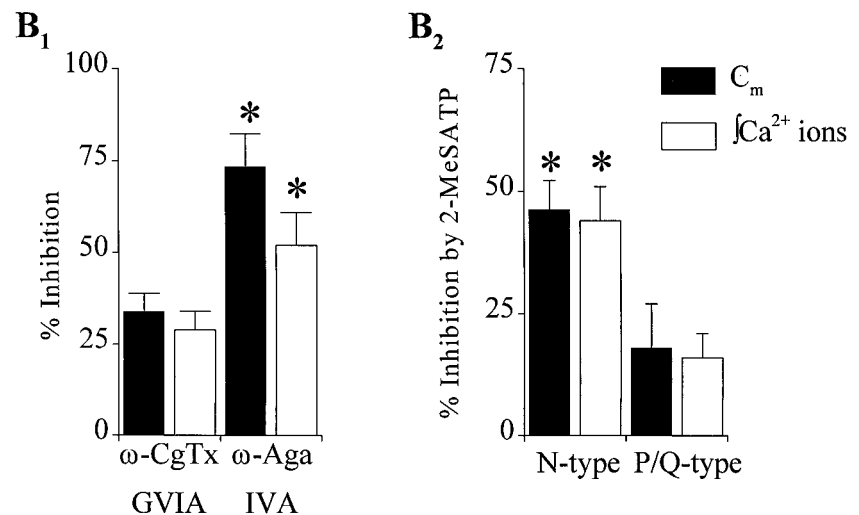
Strong depolarizing prepulses reverse 2-MeSATP inhibition of ΔC_m

Modulation of neuronal Ca^{2+} channels by $G_{i/o}$-proteins is thought to occur via a "membrane-delimited" mechanism involving a direct interaction of $G_{\beta\gamma}$ subunits with the Ca^{2+} channel α subunit (Dolphin, 1998). This interaction is voltage-dependent and can be relieved by strong depolarizing prepulses. We reasoned that, if 2-MeSATP inhibited exocytosis via inhibition of Ca^{2+} channels, then the inhibition of evoked ΔC_m should also be attenuated by strong depolarizing prepulses. To test this, the test pulse used to evoke exocytosis was preceded by a 20 msec depolarizing pulse to +120 mV (Fig. 4*A,B*). In the absence of the agonist, application of the prepulse had no significant effect on either ΔC_m (98 ± 1% of control) or Ca^{2+} (98 ± 1% of control; *n* = 5; data not shown) entry. In the presence of 2-MeSATP (100 nM), a depolarizing prepulse, however, reduced the inhibition of both Ca^{2+} entry and ΔC_m from 23 ± 3 and 35 ± 4% to 10 ± 2 and 12 ± 4%, respectively (*n* = 9) (Fig. 4*C*). To show that the decreased inhibition by 2-MeSATP observed after a prepulse was not simply a result of receptor desensitization, in three of these cells, superfusion with 2-MeSATP was continued and an addi-

tional test pulse without a prepulse was given. The Ca^{2+} entry and ΔC_m response to this second test pulse were inhibited by 16 ± 3 and 31 ± 7%, respectively (*n* = 3). These experiments demonstrate that the inhibition of exocytosis by 2-MeSATP has the same voltage-dependence as inhibition of Ca^{2+} channels in adrenal chromaffin cells and support the hypothesis that the inhibition of ΔC_m observed is caused by inhibition of Ca^{2+} entry.

P2Y purinoceptor activation does not alter the Ca^{2+}-dependence of ΔC_m

To test further the hypothesis that inhibition of Ca^{2+} entry accounts for inhibition of ΔC_m, we examined the effect of increasing pulse duration on 2-MeSATP inhibition of stimulus–secretion coupling. During long depolarizations, agonist-dependent inhibition of Ca^{2+} channels is overcome (Dolphin, 1998), and therefore, an attenuation of the effects of 2-MeSATP on exocytosis are expected. The time-dependence of the modulation of ΔC_m and Ca^{2+} entry by 2-MeSATP are shown in Figure 5, *A* and *B*. Increasing pulse duration from 20 to 400 msec significantly reduced ($p < 0.01$) the inhibition of both ΔC_m and Ca^{2+} entry from 67 ± 11 and 51 ± 2% (*n* = 5) to 14 ± 4 and 12 ± 3% (*n* =
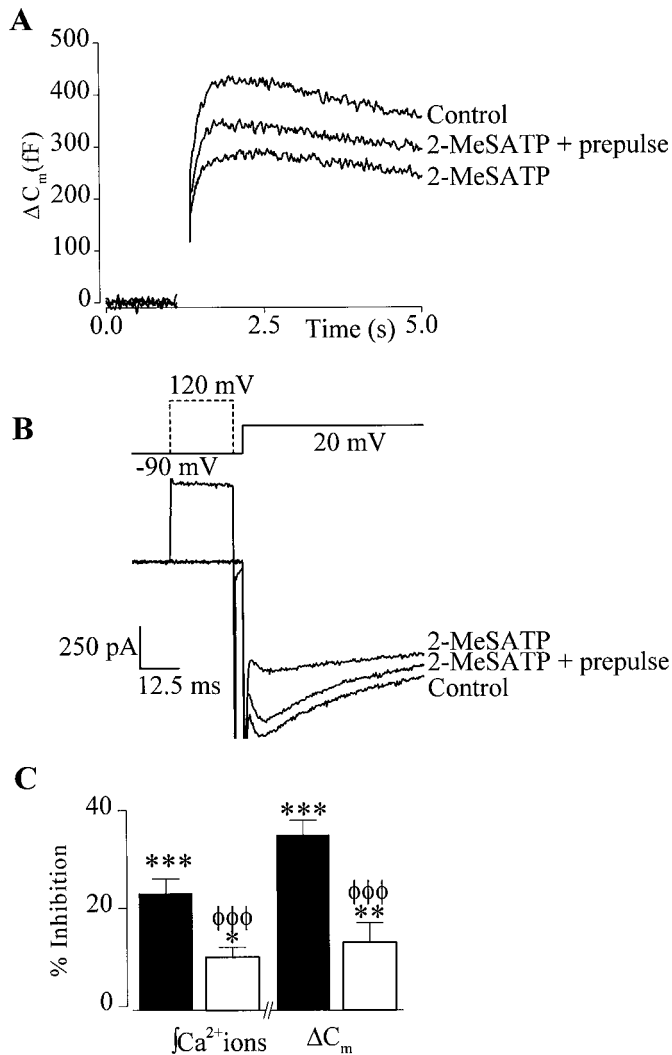
Figure 4. Modulation of both integrated Ca^{2+} entry and ΔC_m was voltage-dependent. *A*, Superimposed traces from a single cell showing the effect of 2-MeSATP on ΔC_m evoked by a 200 msec depolarization to +20 mV from a holding potential of −90 mV with and without a 20 msec depolarizing prepulse. *B, Top*, Schematic diagram of prepulse (*hashed line*) voltage protocol. *B, Bottom*, Superimposed I_{Ca} used to evoke ΔC_m illustrated in *A*. Only the first 100 msec of the 200 msec test pulse are shown for clarity. *C*, Summary of the effects of 2-MeSATP on integrated Ca^{2+} entry and ΔC_m in the presence (*open bars*) or absence (*filled bars*) of a depolarizing prepulse ($n = 9$). Significant effects of 2-MeSATP on Ca^{2+} entry and C_m relative to control are indicated: ***$p < 0.005$; **$p < 0.01$; *$p < 0.05$. Significant differences between responses recorded with and without a prepulse are indicated by $^{\phi\phi\phi}p < 0.005$.

4), respectively. Moreover, the small inhibition remaining with 400 msec depolarization was reversed by depolarizing prepulses to +120 mV ($n = 4$; data not shown). The results from these experiments support the role of Ca^{2+} channels in agonist-dependent inhibition of exocytosis.

Inhibition of exocytosis by a GPCR may also result from a direct decrease in the Ca^{2+} sensitivity of the secretory machinery or a decrease in the probability of release. The concomitant inhibition of ΔC_m and Ca^{2+} entry, coupled with the lack of significant effect of 2-MeSATP on exocytotic efficiency (Fig. 2*E*), provide some evidence that the Ca^{2+} sensitivity of the secretory machinery was not changing. To test this further, we examined the Ca^{2+}-dependence of ΔC_m using the method described by

Engisch and Nowycky (1996). Exocytosis was evoked by single pulses of varying duration, first under control conditions and then in the presence of 2-MeSATP (100 nM) (Fig. 5*A*). The control data from 10 cells was plotted and fit with an allometric function $\Delta C_m = g * (\Delta Ca^{2+})^n$, and the best fit was obtained with a proportionality constant $g = 0.0842$ and power $n = 1.62$ (Fig. 5*C*). The power value obtained by fitting data from individual cells with the allometric function ranged from $n = 1.01$ to 3.49, mean $n = 1.68 \pm 0.29$. Similar variability and values for n were reported by Engisch and Nowycky (1996). What accounts for this variability is at present unknown; however, it may involve differences in the amounts of endogenous Ca^{2+} buffers and size of the readily releasable pool of vesicles. Thus, in cells in which endogenous Ca^{2+} buffering is high, the relationship may be more linear because of a requirement to saturate the buffer before exocytosis can proceed. Similarly, in cells in which the size of the readily releasable pool of vesicles is small, the secretory response measured in response to long depolarizations may be dominated by release from the reserve pool. The Ca^{2+}-dependence of vesicle recruitment from the reserve pool to the readily releasable pool is essentially linear (Neher, 1998). None the less, data obtained by varying Ca^{2+} entry by changing the step potential ($n = 4$) (Fig. 5*C, open triangle*) or application of Ca^{2+} channel toxins (data not shown) did not deviate from the allometric fit, demonstrating that the Ca^{2+}-dependence of exocytosis was unaffected by simply reducing Ca^{2+} influx. Similarly, exocytosis evoked by 200 or 400 msec depolarizations in the presence of 2-MeSATP also overlays the control allometric fit (Fig. 5*C, open circles*). These experiments provided evidence that the inhibition of exocytosis observed with 2-MeSATP was not caused by a decrease in the Ca^{2+}-dependence of exocytosis.

2-MeSATP does not modulate exocytosis evoked by flash-released Ca^{2+}

To determine whether 2-MeSATP inhibited the secretory machinery in chromaffin cells, we bypassed Ca^{2+} channels and used flash photolysis of caged Ca^{2+} to provide the trigger for exocytosis. We hypothesized that, if 2-MeSATP decreased either the Ca^{2+}-dependence or the probability of release, then the rate of ΔC_m evoked by photolyzed Ca^{2+} should also be decreased relative to control. In these experiments, we used NP-EGTA as the photolabile Ca^{2+} chelator because, in contrast to (1-(2-nitro-4,5-dimethoxyphenyl)-1,2-diaminoethane-*N,N,N′,N′*-tetra-acetic; dimethoxynitrophenamine)-nitrophen, it did not produce a loading transient and produced smaller increases in $[Ca^{2+}]_i$, thus avoiding exocytosis of nonsecretory granules (Neher and Zucker, 1993; Parsons et al., 1996; Xu et al., 1998). Figure 6*A* shows a typical whole-cell recording of ΔC_m and $[Ca^{2+}]_i$ from a chromaffin cell loaded with NP-EGTA Ca^{2+} and fura-2. After 3 min of dialysis a UV flash was given to elevate $[Ca^{2+}]_i$, which produced a biphasic increase in ΔC_m (Fig. 6*B*). Because the $[Ca^{2+}]_i$ could not be recorded during the flash, the estimated peak $[Ca^{2+}]_i$ was calculated by fitting the decay of the fura-2 signal with a monoexponential curve. The $[Ca^{2+}]_i$ evoked by a single flash measured by this method peaked at 3.7 ± 1.2 μM ($n = 12$), well below the 100 μM $[Ca^{2+}]_i$ reported to evoke nonsecretory granule fusion (Xu et al., 1998). The mean exocytotic rate measured during 1 sec immediately after the flash was 99 ± 14 fF/sec ($n = 9$), significantly less than the rate evoked by a single 200 msec depolarization ($\sim$1000 fF/sec). Previous studies have shown that the rate of secretion is proportional to $[Ca^{2+}]_i$ and the high exocytotic rates typically produced by depolarizations require $[Ca^{2+}]_i > 50$ μM

Figure 5. P2Y receptor activation did not affect the Ca^{2+}-dependence of C_m changes evoked by long depolarizations. *A*, Superimposed traces show the effect of increasing voltage step duration on I_{Ca} (*bottom*) and corresponding ΔC_m (*top*) in the absence and presence of 2-MeSATP ($\Diamond$). *B*, Plot of the inhibition of integrated Ca^{2+} entry ($\square$) and ΔC_m ($\blacksquare$) by 2-MeSATP, for 20 (*n* = 5), 50 (*n* = 5), 200 (*n* = 15), and 400 (*n* = 4) msec step depolarizations to +20 mV. A significant reduction of the effect of 2-MeSATP on integrated Ca^{2+} entry and ΔC_m evoked by longer depolarizations relative to 20 msec are indicated: **p < 0.01; *p < 0.05. *C*, Plot of mean ΔC_m versus mean integrated Ca^{2+} entry for 10 experiments similar to that illustrated in *A*. ▲, Data obtained under control conditions with voltage steps to +20 mV of varied durations. Data were binned according to pulse duration (7.5-500 msec). *Horizontal* error bars represent the SEM of the integrated Ca^{2+} entry for a given pulse duration. ○, Data obtained with 200 and 400 msec voltage steps to +20 mV (*arrows*) in the absence and presence of 100 nM 2-MeSATP. ▽, Data obtained under control conditions but with 200 msec depolarizations to 0 mV. The Ca^{2+}-dependence of ΔC_m was calculated according to the method of Engisch and Nowycky (1996). Mean control data were fit to the equation $\Delta C_m = g * (\Delta Ca^{2+})^n$; the *solid line* through the data represents the best fit with proportionality constant $g = 0.08$ and the power $n = 1.62$. ΔC_m recorded in the presence of 2-MeSATP did not significantly deviate from the control Ca^{2+}-dependence curve.
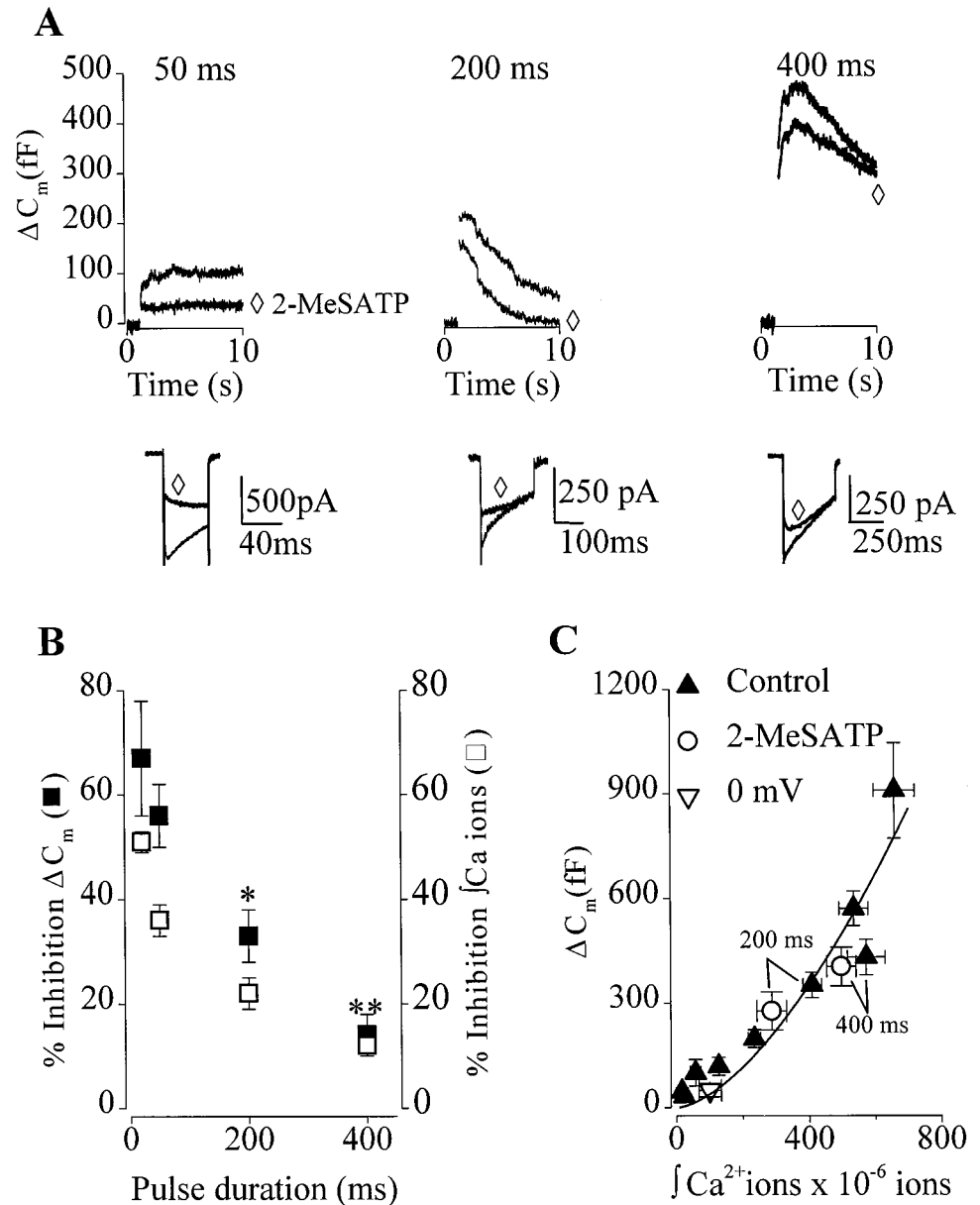
(Neher and Zucker, 1993). Thus, the relatively slow secretory rates observed with photoreleased Ca^{2+} in our studies are consistent with the small increases in $[Ca^{2+}]_i$ measured (~ 4 µM) and are in good agreement with the rates obtained by Heinemann et al. (1994).

Under control conditions, we observed rapid secretory rundown such that subsequent flashes failed to produce reproducible ΔC_m, despite comparable increases in $[Ca^{2+}]_i$ (Fig. 6*A*), suggesting that refilling of the readily releasable pool was impaired in whole-cell recording (see also Seward and Nowycky, 1996). This prevented the examination of the effects of P2Y purinoceptor activation on flash-evoked ΔC_m within a single cell. Therefore, we compared secretory rates recorded from matched (same culture) control cells and cells superfused with 100 nM 2-MeSATP for 2 min before the first UV flash. 2-MeSATP did not significantly affect the rates (Fig. 6*B*), nor did it inhibit the extent of ΔC_m (control, 194 ± 56 fF, *n* = 6; 2-MeSATP, 140 ± 49 fF, *n* = 4) evoked by photolysis of NP-EGTA, providing further evidence

against a direct inhibitory action of P2Y purinoceptors on the secretory machinery of chromaffin cells.

2-MeSATP acts through $G_{i/o}$-proteins to inhibit ΔC_m evoked by a train of depolarizing pulses

In situ chromaffin cells secrete catecholamines, ATP, and peptides most efficiently in response to splanchnic nerve stimulation and bursts of action potentials (Douglas and Poisner, 1966; Edwards et al., 1980). To determine whether P2Y receptor activation inhibited exocytosis evoked by bursts of action potentials, we examined the effects of 2-MeSATP on ΔC_m evoked by trains of short-duration depolarizations. A control train of 20 depolarizations of 20 msec duration given at 2.5 Hz evoked entry of 541 ± 42 × 10^6 Ca^{2+} ions and ΔC_m of 265 ± 31 fF in total, and an overall mean exocytotic efficiency of 0.64 ± 0.2 fF/10^6 Ca^{2+} ions. Similar responses were evoked by subsequent control trains of depolarizations (mean exocytotic efficiency 0.52 ± 0.7 fF/10^6 Ca^{2+} ions; *n* = 23; *p* = 0.92), showing that exocytosis did not
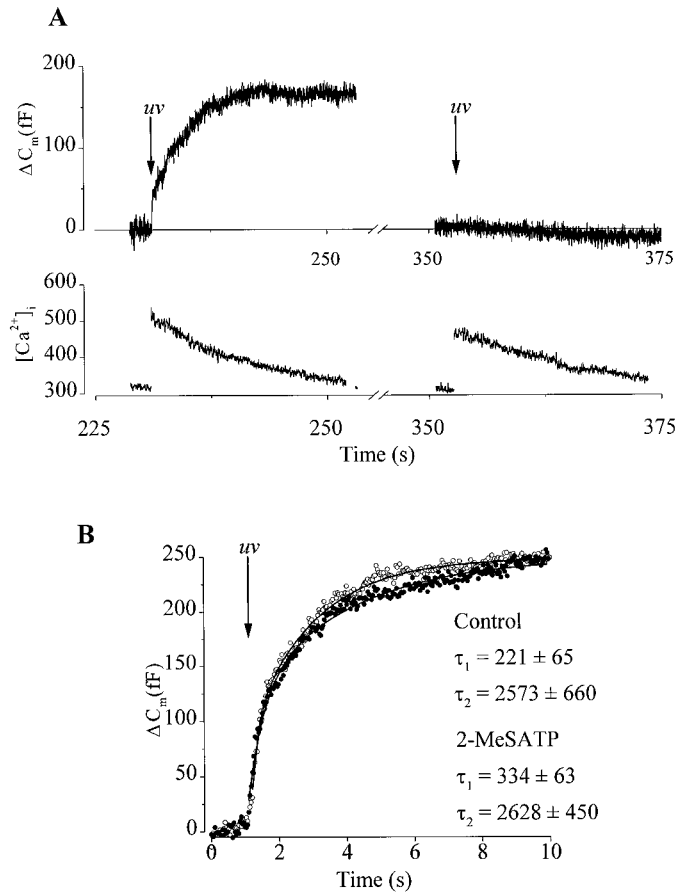
Figure 6. P2Y receptor activation did not modulate secretion evoked by flash photolysis of caged Ca^{2+}. *A*, C_m traces (*top trace*) and corresponding $[Ca^{2+}]_i$ (*bottom trace*) recorded in response to the first flash (80 J, 500 μsec) given ~240 sec after establishing the whole-cell configuration (*left panel*) and a second flash of the same intensity given 120 sec later to the same cell (*right panel*). *B*, Kinetic analysis of two example ΔC_m recorded in response to the first flash after establishing the whole-cell configuration, either under control conditions ($\bigcirc$) or in the presence of 100 nM 2-MeSATP ($\bullet$). For clarity of illustration, only every third point is shown. The *solid line* drawn through the data represents a double-exponential fit obtained using the C_m point immediately after the UV flash and the plateau level as limits. The mean time constants displayed are for both control conditions ($n = 5$) and in the presence of 100 nM 2-MeSATP ($n = 4$).

undergo activity-dependent changes with these relatively mild stimuli (Engisch et al., 1997; Smith, 1999). Both the ΔC_m and Ca^{2+} entry in response to a train of depolarizations were significantly inhibited by 100 nM 2-MeSATP (Fig. 7*A,B*); thus, total Ca^{2+} entry was reduced to $435 \pm 34 \times 10^6$ Ca^{2+} ions and total ΔC_m to 222 ± 31 fF ($n = 23$). Inhibition of Ca^{2+} entry and ΔC_m were, however, more pronounced early in the train, reaching a maximum of $54 \pm 6\%$ on the fourth pulse and a minimum of $18 \pm 5\%$ on the 20th pulse ($n = 23$) (Fig. 7*B*).

Neither the exocytotic efficiency (0.51 ± 0.6 fF/10^6 Ca^{2+} ions; $p = 0.98$) nor the Ca^{2+}-dependence of secretion evoked by trains of depolarizations was altered by 2-MeSATP (Fig. 7*C*), suggesting that the mechanisms involved in inhibition of secretion evoked by single long pulses or trains of depolarizations are similar. We next examined the voltage-dependence of the inhibition by giving a 20 msec depolarizing prepulse to +120 mV before every test pulse in the train. In the absence of depolarizing prepulses, 2-MeSATP inhibited both Ca^{2+} entry and ΔC_m by 35 ± 4 and $45 \pm 8\%$,
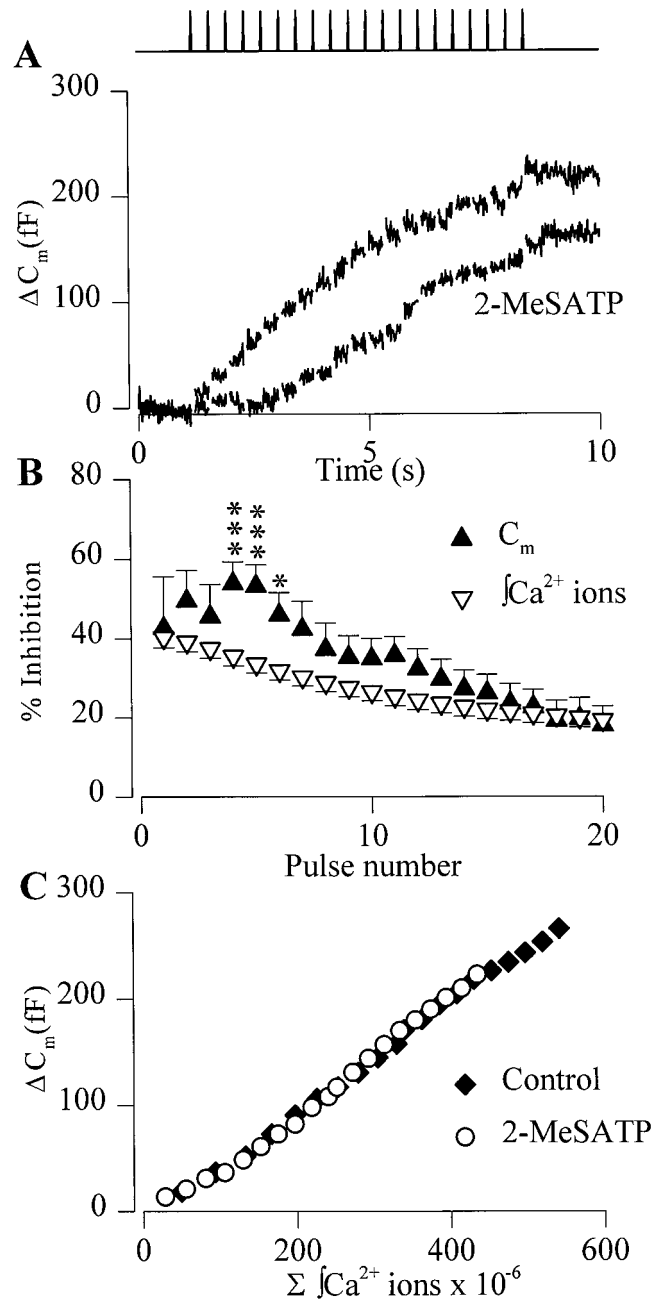

Figure 7. P2Y receptor activation inhibited ΔC_m evoked by a train of depolarizing pulses. *A*, Superimposed C_m traces recorded from a single cell in response to a train of twenty 20 msec depolarizing pulses to +20 mV from −90 mV. A control train (*top trace*) evoked an appreciable change in C_m, and superfusion of 2-MeSATP (*bottom trace*) inhibited the ΔC_m in response to a subsequent train. Gaps in the traces and schematic in the *top panel* indicate timing of the pulses. *B*, Summary of the mean $\pm$ SEM inhibition of integrated Ca^{2+} entry ($\triangledown$) and ΔC_m ($\blacktriangle$) by 2-MeSATP for each pulse in the train of depolarizations. Significant differences between inhibition of integrated Ca^{2+} entry and ΔC_m changes are shown: ***$p < 0.005$; *$p < 0.05$ ($n = 23$). *C*, Plot of the mean Ca^{2+}-dependence of ΔC_m measured in response to a train of depolarizing pulses in the absence ($\blacklozenge$) and presence ($\bigcirc$) of 2-MeSATP ($n = 23$). Error bars are omitted for clarity.

respectively (mean inhibition for pulses four to eight of a train, $n = 4$). In the same cells, a depolarizing prepulse preceding each test pulse reduced the inhibitory effect of 2-MeSATP on Ca^{2+} entry to $-2 \pm 4\%$ and ΔC_m to $-17 \pm 12\%$. Finally, we examined the

second messenger system mediating the inhibitory effects of 2-MeSATP on stimulus–secretion coupling in chromaffin cells. Pretreatment with PTX (250 ng/ml, 24 hr) reduced the inhibitory effect of 2-MeSATP (100 nM) on Ca^{2+} entry to $-2 \pm 2.5\%$ and ΔC_m to $-8 \pm 14\%$ ($n = 4$). Collectively, these data show that 2-MeSATP inhibition of exocytosis evoked by single or trains of depolarizations was mediated by a $G_{i/o}$-coupled P2Y purinoceptor modulating Ca^{2+} entry through Ca^{2+} channels.

DISCUSSION

Given the ubiquity of ATP in secretory vesicles (Zimmermann, 1994) and the profusion of P2 purinoceptors in the nervous system (North and Barnard, 1997), the potential for ATP to play a major modulatory role in neurotransmission is high. In the sympathetic nervous system, the inhibitory effects of presynaptic purinoceptors on neurotransmission have been well documented (Von Kügelgen et al., 1989; Boehm, 1999). A lack of selective agonists and antagonists, coupled with the inaccessibility of the majority of mammalian nerve terminals, have, however, made investigation of the mechanisms underlying inhibition of transmitter release by ATP difficult. In this study, we have used combined voltage-clamp recording and C_m measurements to investigate the effects of purinoceptors on exocytosis in adrenal chromaffin cells. The results show that 2-MeSATP, a potent agonist at P2Y purinoceptors, inhibited exocytosis through an effect on N- and P/Q-type Ca^{2+} channels. The inhibition of stimulus–secretion coupling was mediated by $G_{i/o}$-proteins and was voltage-dependent. Activation of P2Y purinoceptors had no effects downstream of Ca^{2+} entry on the secretory machinery. The results of this study strongly support the hypothesis that inhibition of presynaptic Ca^{2+} channels plays the major role in presynaptic inhibition of elicited neurotransmitter release by $G_{i/o}$PCRs (Wu and Saggau, 1997) and that this is the mechanism underlying negative feedback modulation of sympathetic transmitter release by P2Y purinoceptors.

Inhibition of neuronal Ca^{2+} channels by GPCR is thought to represent a ubiquitous mode of modulation of neuronal function (Hille, 1994). The most widely studied and best understood pathway is a membrane-delimited pathway involving a direct interaction of $G_{\beta\gamma}$ subunits with the Ca^{2+} channel pore-forming α_1 subunit (Page et al., 1998; Jeong and Ikeda, 1999). A characteristic property of this signaling pathway is its voltage-dependence in that the inhibition is relieved by strong depolarizations (Dolphin, 1998). In agreement with previous studies in chromaffin cells (Diverse-Pierluissi et al., 1991; Currie and Fox, 1996), we show that P2Y purinoceptors inhibit N- and P/Q-type Ca^{2+} channels by a PTX-sensitive, voltage-dependent pathway, strongly suggesting that the membrane-delimited pathway is involved (Figs. 1, 3, 4). A functional link between the P2Y-mediated suppression of evoked I_{Ca} and secretion is shown by the inhibition of evoked ΔC_m (Figs. 2, 3, 6). In agreement with previous studies (Currie and Fox, 1997; Zamponi and Snutch, 1998), we also find a greater inhibition of Ca^{2+} entry through N-type over P/Q-type channels, and this is paralleled by a greater inhibition of secretion supported by N-type channels. Paradoxically, we found that N-type channels contributed less than P/Q-type channels to secretion evoked by long depolarizations. The lower sensitivity of P/Q-type channels to modulation by 2-MeSATP suggests either a lower efficacy of interaction between $G_{i/o}$-proteins and P- and/or Q-type channels (Bourinet et al., 1999) or preferential compartmentalization of P2Y purinoceptors with N-type channels (Carabelli et al., 1998). Such differential coupling between a GPCR and exocytosis will have

significant functional consequences in neurons in which there is tight coupling between subtypes of Ca^{2+} channels and transmitter release (for review, see Caterall, 1999) and could provide a mechanism whereby cotransmitters stored in separate pools of vesicles may be independently modulated (Von Kügelgen, 1996).

The results from this study demonstrate that the inhibition of exocytosis by a $G_{i/o}$PCR can be fully accounted for by the inhibition of Ca^{2+} channels. Three lines of evidence support this hypothesis. First, the Ca^{2+}-dependence of secretion for both stimulus parameters is unaffected by activation of P2Y purinoceptors (Figs. 5, 7). Second, strong depolarizing prepulses relieve the voltage-dependent inhibition of Ca^{2+} channels by 2-MeSATP and, in parallel, the inhibition of exocytosis (Fig. 4). We do not believe that the voltage-dependent relief of secretory inhibition observed in chromaffin cells is caused by a Ca^{2+}-independent, voltage-dependent enhancement of exocytosis similar to that described for fast neurotransmitter systems (Linial et al., 1997; Mochida et al., 1998) because, under our control conditions, strong depolarizing prepulses did not enhance ΔC_m per se. The final piece of evidence for the lack of effect of P2Y purinoceptor activation on the secretory machinery comes from the flash photolysis experiments in which exocytosis was evoked independently of Ca^{2+} channels (Fig. 6). Our data contradicts conclusions from a previous study in which it was suggested that ATP can inhibit the exocytotic machinery in chromaffin cells (Lim et al., 1997). The discrepancy between the two studies may arise from either differences between signaling systems of chromaffin cells from two species or differences in the methods used. Lim and coworkers (1997) used the whole-cell patch-clamp recording technique and examined the rates of exocytosis after slow infusion of buffered Ca^{2+} solutions into the cells. Differences in chromaffin cell secretory properties have been reported with secretory rundown, complicating data interpretation (von Ruden and Neher, 1993; Seward et al., 1995; Engisch and Nowycky, 1996). Interestingly, the results from our study are in close agreement with results from a central synapse (Takahashi et al., 1996) and peptidergic terminals (Rusin et al., 1997), suggesting that G-protein regulation of voltage-operated Ca^{2+} channels is the predominant mechanisms underlying presynaptic inhibition of evoked release.

Exocytosis evoked by long depolarizations in chromaffin cells consists of two kinetically distinct components, referred to as immediate and slow exocytosis (Horrigan and Bookman, 1994). P2Y purinoceptor activation inhibited the slow phase of exocytosis to a significantly greater extent than the immediate phase (Fig. 2). A similar observation has been previously reported for opioid inhibition of exocytosis in the neurohypophysis (Rusin et al., 1997). The two kinetically distinct phases of exocytosis are thought to represent release of vesicles with varying degrees of readiness depending on their location in the secretory pathway; thus, immediate exocytosis is caused by fusion of readily releasable vesicles docked to Ca^{2+} channels, and slow release is caused by fusion of readily releasable vesicles responding to diffused Ca^{2+} (Thomas et al., 1990; Horrigan and Bookman, 1994; Chow et al., 1996; Seward and Nowycky, 1996). Alternatively, slow release may represent exocytosis of vesicles from the reserve pool, which are also regulated by diffused Ca^{2+}. Release evoked by diffused Ca^{2+} is expected to involve multiple subtypes of Ca^{2+} channels and their associated $G_{i/o}$-proteins, and therefore, consistent with our results, the inhibition of slow release should be correlated to the inhibition of the total Ca^{2+} entry. On the other hand, if immediate ΔC_m are associated with chromaffin granules preferentially colocalized with a subtype of Ca^{2+} channel, then modulation of immediate secretion would depend on the

efficacy of coupling between $G_{i/o}$-proteins and that particular channel subtype. The relatively small inhibition of immediate exocytosis observed with 2-MeSATP more closely matches the inhibition of P/Q-type than N-type Ca^{2+} channels, which would be expected if granules are preferentially docked to P/Q-type channels. Support of granule docking to P/Q-type channels comes from a previous study (Lara et al., 1998), as well as our own observations that ω-Aga IVA is a more potent inhibitor of exocytosis than ω-CgTx GVIA. Alternatively, a reduced effectiveness of P2Y purinoceptors to inhibit immediate exocytosis may result from saturation of the Ca^{2+} sensor (Rusin et al., 1997). We do not believe this to be the case because, if the fusion machinery were saturated then at short depolarizations in which release occurs predominantly from the readily releasable pool, the inhibition of secretion by 2-MeSATP should be occluded. This was not observed; indeed, inhibition was greatest with short depolarizations (Fig. 5B). Although the mechanisms underlying the reduced inhibition of immediate release require further investigation, a consequence of this phenomenon will be that, *in vivo*, released ATP would be more effective at inhibiting asynchronous release than synchronous release and thus would serve to maintain a tight temporal correlation between action potential firing and evoked transmitter release.

Catecholamine secretion from chromaffin cells is most efficiently triggered by high-frequency stimulation of the splanchnic nerve and bursts of action potentials (Edwards, 1982; Zhou and Misler, 1995). We found that P2Y modulation of secretion declines during a train of short depolarizations such that ~50% of the modulatory effect remains at the end of a train. There are two possible explanations for the loss of effect. First, this study (Fig. 7B) and other studies (Seward et al., 1995; Engisch and Nowycky, 1996) show that significant inactivation of Ca^{2+} channels occurs during a train of depolarizations. It is possible that the loss of modulatory effect is caused by inactivation of the Ca^{2+} channels modulated by 2-MeSATP. Alternatively, recent studies have shown that voltage-dependent modulation of Ca^{2+} channels by $G_{\beta\gamma}$ subunit is strongly dependent on the frequency at which the channels are activated, with high-frequency trains reversing inhibition (Brody et al., 1997; Park and Dunlap, 1998). It seems unlikely, however, that this is occurring in our experiments because reinhibition of N- and P/Q-type channels by G-proteins has a time constant of ~100 msec in chromaffin cells (Currie and Fox, 1997), which is significantly faster than the 400 msec interpulse duration used in our studies. Therefore, at the high stimulus frequencies that can occur *in vivo* when an animal is in danger and the fight-or-flight response is activated, purinoceptor inhibition of catecholamine secretion may be virtually abolished by a combination of these two mechanisms.

Chromaffin cells costore and secrete various neuromodulators with catecholamines, including ATP (Winkler and Westhead, 1980). The functional role of ATP in secretory vesicles has recently become the focus of much interest. Evidence suggesting that ATP forms part of an autocrine inhibitory loop controlling Ca^{2+} channels has been provided by several studies (Albillos et al., 1996; Currie and Fox, 1996; Carabelli et al., 1998). The results of this study show that P2Y purinoceptors inhibit stimulus-evoked exocytosis in chromaffin cells as a direct consequence of $G_{i/o}$-protein inhibition of Ca^{2+} channels and the Ca^{2+} signals regulating vesicle fusion rather than a direct effect on the exocytotic machinery. Presynaptic P2Y purinoceptors in the brain and periphery may act in a similar manner to regulate exocytosis of catecholamines, as well as other transmitters.

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