J. Membrane Biol. 122, 23–31 (1991)
00222631910007 7P

The Journal of
Membrane Biology

Pertussis Toxin Stimulation of Catecholamine Release from Adrenal Medullary Chromaffin Cells: Mechanism May Be by Direct Activation of L-Type and G-Type Calcium Channels

V. Ceña*, K.W. Brocklehurst, H.B. Pollard, and E. Rojas
Laboratory of Cell Biology and Genetics, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Summary. We have previously shown that pertussis toxin (PTX) stimulates delayed-onset, $[Ca^{2+}]_o$-dependent catecholamine (CA) release from bovine chromaffin cells. We now show that this effect of PTX is inhibited in part (50%) by dihydropyridine Ca^{2+}-channel antagonists niludipine and nifedipine, and is potentiated by the dihydropyridine Ca^{2+}-channel agonist Bay K-8644. We and others have shown that pretreatment of chromaffin cells with PTX results in enhanced catecholamine secretion in response to high $[K^+]_o$, nicotine and muscarine, and here we extend these observations by showing that toxin pretreatment also enhances the secretory response to $[Ba^{2+}]_o$. All these data are consistent with the concept that PTX may act on Ca^{2+} channels. To examine the possibility of a direct action of the toxin on the voltage-gated L-type Ca^{2+} channel known to be present in these cells, we studied the effects of the toxin on whole cell Ca^{2+} currents. We found and report here that spontaneous electrical activity was considerably increased in PTX-treated cells. Our measurements of whole cell inward Ca^{2+} currents indicate that the underlying mechanism is a marked shift of the activation curve of the L-type Ca^{2+} current along the voltage axis towards more negative potentials. While treatment of the cells with PTX had no effect on L-type Ca^{2+}-channel conductance (6 nS/cell at 2.6 mM $[Ca^{2+}]_o$), PTX evoked the activation of a new class of Ca^{2+}-selective channels (5 pS in 25 mM $[Ca^{2+}]_{pipet}$), which are rather insensitive to membrane potential. We have termed these *G-type* calcium channels. These data suggest that treatment with PTX not only increases the probability of L-type Ca^{2+}-channel activation at more negative potentials, but also increases the probability of opening of an entirely new, voltage-independent, Ca^{2+} channel. These actions of PTX should promote Ca^{2+} entry and might explain the stimulation by the toxin of CA secretion from medullary chromaffin cells in culture.

Key Words catecholamine secretion · medullary chromaffin cell · pertussis toxin · G-type Ca^{2+} channel · L-type Ca^{2+} channel · G-protein

Introduction

Pertussis toxin stimulates CA release from cultured bovine chromaffin cells in the absence of any exogenous stimulatory or inhibitory agonists for this cell (Brocklehurst & Pollard, 1988). The effect of the toxin was only apparent after an approximately 2 hr time lag, although exposure times as short as 15 min were sufficient for the effect of the toxin to develop. The effect of the toxin was also dependent on the presence of extracellular $[Ca^{2+}]_o$, and was accompanied by the ADP ribosylation of an approximately 40,000-M_r protein(s).

If it is assumed that PTX is acting through a GTP-binding protein(s) (G-protein) to mediate its effect on catecholamine release, there are several stages of the secretory process where such a G-protein may be involved. G-proteins have been shown to regulate ion channels in the plasma membrane (Brown & Birnbaumer, 1988), to be involved in regulation of intracellular membrane fusion (for review *see* Bourne, 1988), and have been postulated to be involved directly in the process of exocytosis (Cockcroft, Howell & Gomperts, 1987).

Since the common mechanism of action of many secretagogues of bovine chromaffin cells involves an elevation of the cytosolic free Ca^{2+} concentration (Pollard et al., 1985), we investigated the possibility that PTX toxin might act on the L-type voltage-gated Ca^{2+} channel known to be present in the membrane of medullary chromaffin cells (Ceña, Stutzin & Rojas, 1989). We found that, although the conductance due to the activation of the L-type Ca^{2+} channel was not affected in chromaffin cells treated with PTX, the activation curve of the L-type Ca^{2+} current was shifted along the voltage axis towards more negative potentials. Furthermore, we found that the toxin activated a small conductance G-type Ca^{2+} channel, hitherto not previously detected in chromaffin cells. Our results suggest that PTX activates secretion from chromaffin cells by modification of two distinct Ca^{2+} conductance processes.

* *Present address:* Division of Pharmacology, Department of Neurochemistry, Faculty of Medicine, University of Alicante, Alicante, Spain.

Materials and Methods

Preparation and Culture of Chromaffin Cells

Primary cultures of bovine adrenal chromaffin cells were prepared as described elsewhere (Greenberg & Zinder, 1982). Cells were maintained in culture using either 24-well cluster plates (Costar, Cambridge, MA) at a density of 10^6 cells/1.5 ml per 2 cm^2 for catecholamine release experiments or in 35-mm diameter dishes (Costar) at a density of 10^6 cells/ml in 2 ml of culture medium for electrophysiological measurements (Brocklehurst & Pollard, 1988). Cells were maintained in culture for 2–4 days before experiments. Pertussis toxin (List Biologicals, Campbell, CA) was suspended in water at a concentration of 100 μg/ml and subsequently diluted to a final concentration of either 100 or 300 ng/ml in DME/F12 culture medium containing 15 mM HEPES and L-glutamine but without phenol red as supplied by Sigma (St. Louis, MO), supplemented with 26 mM $NaHCO_3$ and adjusted to pH 7.3. This modified DME/F12 medium was used for all catecholamine secretion studies. Catecholamine release was measured as described previously (Brocklehurst, Morita & Pollard, 1985) and expressed as a percentage of total cellular catecholamine. All incubation volumes were 0.5 ml, and incubations were performed at 37°C in a CO_2 incubator. In the patch-clamp experiments the cells were cultured for 1–2 days and then incubated for 5–10 hours with pertussis toxin added to the culture medium at a final concentration of 100 ng/ml.

Patch-Clamp Experiments

The methods used have been described in detail elsewhere (Ceña et al., 1989). The pipettes used for patch-clamp experiments were prepared from micro-hematocrit glass tubes (i.e., 1.1–1.2 mm; wall thickness, 0.2 mm). Patch electrodes were coated to near the tip with Sylgard (184 Elastomer kit, Dow Corning, Midland, MI), and the tips were fire polished. After these manipulations patch electrodes had an open tip resistance in the range from 10 to 14 MΩ for cell-attached and from 2 to 4 MΩ for the whole-cell configurations, respectively. Under the experimental conditions used here seals formed spontaneously on most occasions when positive pressure was removed from the pipette interior. The resistance of the seals varied between 15–30 GΩ.

Membrane currents were recorded using an EPC-7 patch-clamp amplifier (List-Electronics, Darmstadt-Eberstadt, FRG). For patch-clamp experiments the culture medium was removed from the cells and the cells were washed several times with a physiological saline (in mM: 140 NaCl, 5 KCl, 2.5 $CaCl_2$, 10 Na-HEPES, pH 7.4). To observe and record spontaneous action potentials, the pipette was filled with either a high K^+ solution (in mM: 140 KCl, 1 $MgCl_2$, 10 Na-HEPES, pH 7.4) or with a high Ca^{2+} solution (in mM: 25 $CaCl_2$, 140 tetramethylammonium (TMA), 5 TMA-HEPES, pH 7.4). Spontaneous electrical activity of the cells and single Ca^{2+}-channel current records were made in the cell-attached configuration on a VCR tape using a PCM/VCR system (Pulse Code Modulator, Sony 501-ES). Whole-cell membrane current records were made on digital magnetic tape using a computer system provided of 12-bit analog-to-digital converter as described elsewhere (Ceña et al., 1989). For patch-clamp experiments in the whole-cell configuration, the culture medium was removed from the cells and the cells were washed several times with a Na-free KRB solution (in mM: 140 cholineCl, 5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 Na-HEPES, pH7.4). The pipette was filled with a K^+-free solution (in mM: 65 CsCl, 75 CsAspartate, 1 $CaCl_2$, 1 $MgCl_2$, 11 Cs-EGTA, 2.5 Mg-ATP, 10 Na-HEPES, pH 7.2). This combination of solutions was chosen to minimize both inward Na^+ current and outward K^+ current. The tip of the pipette was brought close to the cell, and a seal was formed in the cell-attached configuration immediately after the release of the positive pressure from inside the pipette. The capacitance of the pipette was compensated for using the patch-clamp amplifier circuit and the potential in the solution inside the pipette was made −80 mV more negative than the solution bathing the cells. At this point single channel activity was observed (probably Ca^{2+}-activated K^+ channels), and this activity was taken as an index of cell-attached configuration. The patch under the pipette was broken by applying a slight negative pressure inside the pipette. The transition from the cell-attached to the whole-cell recording mode was indicated by the disappearance of the single channel activity and the appearance of a capacity transient due to capacitance of the cell membrane (range from 6 to 8 pF). After waiting for 3 min to ensure sufficient dialysis of the intracellular medium (Neher & Almers, 1986), a series of voltage-clamp pulses increasing in 10-mV steps from −80 up to a membrane potential of 60 mV were applied using a mini-computer (Ceña et al., 1989: Stutzin et al., 1989). The current transients in response to each pulse were digitized at 70 μsec per sample, and 3072 samples per record were stored on digital tape.

For analysis, each record of the current transient representing the membrane response to a given voltage pulse was obtained by averaging two or three responses to the same pulse. Correction for the linear components of the leakage and capacity currents was achieved by subtracting from each record the scaled value of a current transient in response to a pulse which did not elicit Ca^{2+} currents. Then the corrected record was condensed by taking the mean of three consecutive samples and further digitally filtered (corner frequency set at 1–3 kHz). The corrected records were used to generate the current-voltage relationship (Ceña et al., 1989; Stutzin et al., 1989).

Analysis of the Whole-Cell Calcium Currents

Each membrane current record in response to pulses to a particular voltage was fitted using a modified version of the Hodgkin-Huxley empirical model as explained elsewhere (Ceña et al., 1989)

$$i_{Ca}(V,t) = i_{max}(V)\{1 - \exp[-t/\tau_m(V)]\}^2. \quad (1)$$

This analysis generated values for both the maximal current $i_{max}(V)$ and the time constant for activation of the current $\tau_m(V)$ at each membrane potential V.

To calculate the steady-state distribution of the hypothetical Ca^{2+} channel gates m_∞ as a function of V, we estimated first permeability values $P(V)$ from the corresponding $i_{max}(V)$ values using the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin & Katz, 1949). A computer program calculated the reversal potential for the Ca^{2+} current to give a sigmoidal saturating activation curve. Next, m_∞ values obtained by taking the square root of the normalized $P(V)$ values were fitted to the following equation:

$$m_\infty(V) = 1/\{1 + \exp[z_e(V - V_a)/kT]\} \quad (2)$$

where z_e represents the effective valence of the voltage sensor and V_0 the midpoint potential. The activation and deactivation rate constants of the gating system controlling the state of the Ca^{2+} channel (α and β, respectively) were obtained by solving the following equations:

$$m_\infty(V) = \alpha(V)/(\alpha(V) + \beta(V)) \quad (3a)$$

and

$$\tau_m(V) = 1/(\alpha(V) + \beta(V)). \quad (3b)$$

The time constant $\tau_m(V)$ was obtained from the least squares fit of the empirical model given by Eq. (1) to the actual current record (Ceña et al., 1989). Values for the rate constants $\alpha(V)$ and $\beta(V)$ were obtained by the simultaneous solution of Eqs. (3a) and (3b). These values were then fitted to the following equations:

$$\alpha(V) = A \exp[a(V - V_0)/kT] \quad (4a)$$

$$\beta(V) = B \exp[b(V - V_0)/kT] \quad (4b)$$

where A and a (or B and b) represents empirical coefficients with units of sec^{-1} and electronic charges, respectively (Ceña et al., 1989). Time constant values $\tau(V)$ for activation of the current were fitted to the following equation obtained from Eqs. (3b), (4a) and (4b):

$$\tau_m(V) = 1/\{A \exp[a(V - V_0)/kT] + B \exp[b(V - V_0)/kT\}. \quad (4c)$$

Results

Partial Inhibition of PTX-Induced CA Secretion by Dihydropyridine Calcium Channel Antagonists

Chromaffin cells were pretreated with PTX for 2 hr and then incubated for a subsequent 3 hr period in DME/F12 medium with various concentrations of the dihydropyridine Ca^{2+} channel antagonists niludipine (Fig. 1; upper, left side) and nifedipine (Fig. 1; upper, right side) and the agonist Bay-K-8644 (Fig. 1; lower panel) and in the absence of PTX. The CA released into the DME/F12 medium during these 3-hr incubations was measured and the results can be seen in Fig. 1. In this series of experiments, cells pretreated with PTX showed a subsequent three- to fourfold increase in catecholamine release compared to control cells receiving no pretreatment. Niludipine had no effect on the CA released from control cells but inhibited catecholamine release from the toxin-pretreated cells (Fig. 1, upper left). The inhibitory effect of niludipine was apparent at the lowest concentration tested of 0.3 μM with a further small increase in the effect on increasing the concentration up to 3 μM. The maximal extent of inhibition of PTX-induced release in the experiment shown is 59% at 3 μM niludipine, and in identical experiments performed on three different cell preparations the maximal inhibition observed was 30, 58 and 58%. Essentially similar results to those observed with niludipine were obtained with nifedipine (Fig. 1; upper right). In the experiment shown the maximal inhibition of PTX-induced catecholamine release seen at 3 μM nifedipine was 51%, and in three identical experiments performed on three different cell

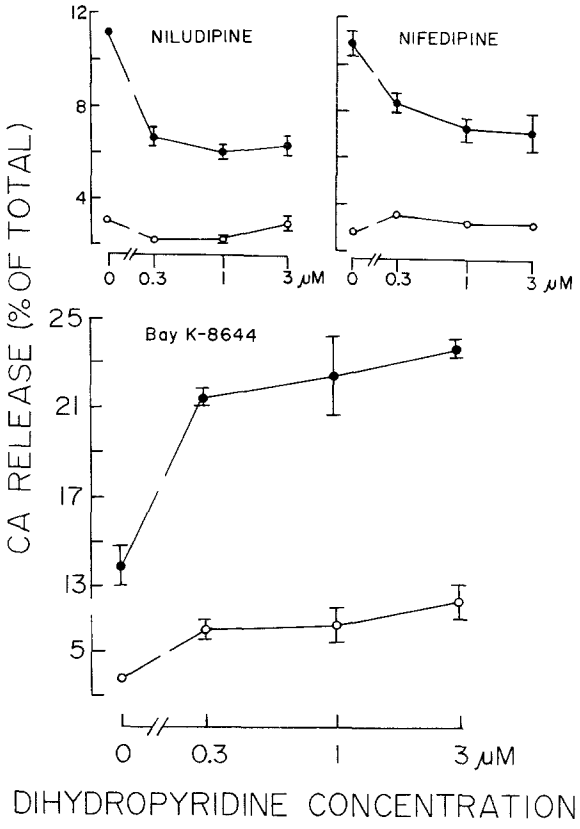

Fig. 1. Effect of dihydropyridine drugs on pertussis toxin-induced catecholamine release. Chromaffin cells were preincubated for 2 hr in the absence (○) or presence (●) of pertussis toxin (100 ng/ml). The medium was then removed, and the cells were incubated for 3 hr in the presence of various concentrations of niludipine (upper left), nifedipine (upper right) or Bay-K-8644 (lower panel) in the absence of PTX. This medium was then removed and assayed for catecholamines and the release expressed as a percentage of total cellular catecholamines. In each case values shown are means ± SD (n = 3) for a representative experiment. Toxin-induced catecholamine release for each condition was also calculated and expressed as a percentage of toxin-induced release in the absence of any dihydropyridine drug. Note the lack of effect of L-type Ca^{2+}-channel antagonists niludipine and nifedipine on basal CA release in the absence of PTX. In contrast, Bay K-8644 stimulates basal CA release. These results suggest that L-type Ca^{2+}-channel activity under resting, nonstimulated conditions is insufficient to contribute to basal CA secretion

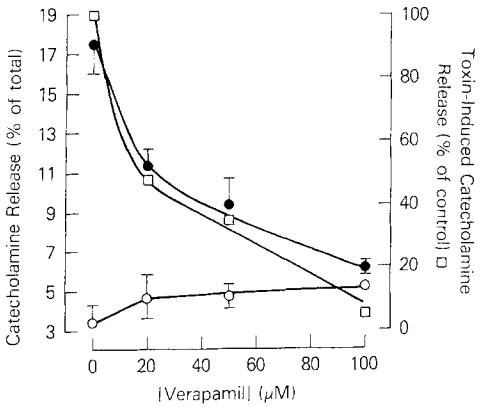

Fig. 2. Effect of verapamil on pertussis toxin-induced catecholamine release. Chromaffin cells were treated as described in the legend to Fig. 1 except that verapamil (●) at the indicated concentrations replaced the dihydropyridine drugs

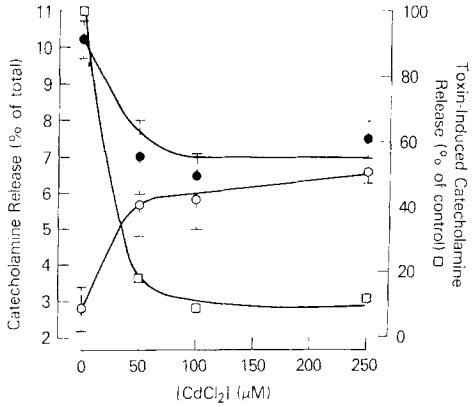

Fig. 3. Effect of Cd^{2+} on pertussis toxin-induced catecholamine release. Chromaffin cells were treated as described in the legend to Fig. 1 except that $CdCl_2$ at the indicated concentration replaced the dihydropyridine drugs (●)

preparations the maximal extent of inhibition was 51, 59 and 69%. In contrast to the results obtained with the dihydropyridine Ca^{2+}-channel blockers, Bay K-8644 was found to potentiate both basal and PTX-induced catecholamine release (Fig. 1, lower panel). This effect of Bay K-8644 was largely apparent at a concentration of 0.3 μM with a further small increase in the effect up to a concentration of 3 μM. The maximal potentiation of toxin-induced release in the experiment shown was 162%, but in two identical experiments performed on two different cell preparations values of 194 and 320% were obtained.

Verapamil and Cadmium Effectively Block PTX-Induced CA Secretion

Similar experiments were performed with two additional Ca^{2+}-channel blockers, namely verapamil (Fig. 2) and Cd^{2+} (Fig. 3). Verapamil induced a slight increase in basal CA release (○) and a large inhibition of catecholamine release from cells pretreated with PTX (●). In the experiment shown in Fig. 2, a 94% inhibition of toxin-induced release was observed with 100 μM verapamil (●). In two identical experiments performed on two different cell preparations the extent of inhibition of PTX-induced CA release by 100 μM verapamil was 97 and 99%. Cd^{2+} at concentrations smaller than 250 μM caused a significant increase in basal CA release (○) whereas the release from PTX pretreated cells was inhibited (●). Thus, in the experiment shown in Fig. 3 a maximal inhibition of 91% of toxin-induced catecholamine release was apparent at 100 μM Cd^{2+} (●). In two identical experiments performed on two different cell preparations the extent of inhibition of toxin-induced catecholamine release by 250 μM Cd^{2+} was 100 and 89%.

Barium Enhances PTX-Evoked Catecholamine Release

Inasmuch as Ba^{2+} enters chromaffin cells through various pathways including voltage-gated Ca^{2+} channels (Forsberg & Pollard, 1988; Heldman et al. 1989), we investigated whether PTX pretreatment of chromaffin cells sensitized them to stimulation by Ba^{2+}. Cells were incubated in the absence or presence of toxin for 3 hr, the medium was removed and the CA released during a 10-min incubation with various concentrations of $BaCl_2$ measured. As shown in Fig. 4, in the absence of Ba^{2+} the release from control (○) and PTX-pretreated cells (●) was the same, but at all Ba^{2+} concentrations used (≤5 mM), Ba^{2+}-induced catecholamine release was enhanced from cells pretreated with toxin. This effect was most marked at lower Ba^{2+} concentrations.

To further understand the mechanism involved in pertussis toxin action, we also examined the effect of treating chromaffin cells with PTX (100 ng/ml) for a period of 5–10 hr on (i) spontaneous electrical activity, (ii) single Ca^{2+}-channel currents (Rojas et al., 1990), and (iii) whole-cell Ca^{2+} currents.

Pertussis Toxin Stimulates Electrical Activity

Our cultured nonstimulated chromaffin cells exhibit spontaneous action potentials (Fig. 5, control records). This electrical activity can be recorded from cell-attached patches using either a high K^+ or a

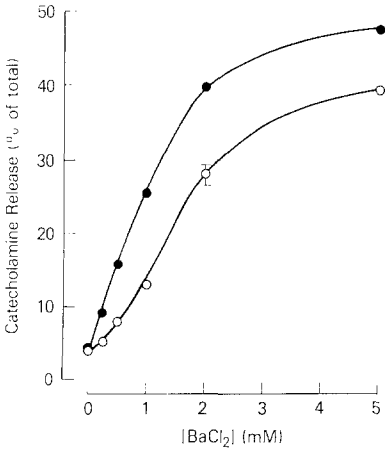

Fig. 4. Effect of pertussis toxin-pretreatment on Ba^{2+}-induced catecholamine release. Chromaffin cells were preincubated for 3 hr in the absence (○) or presence (●) of pertussis toxin (300 ng/ml). The medium was then removed, and the cells were incubated for 10 min in the presence of various concentrations of $BaCl_2$. Catecholamine content in the medium was determined and the release expressed as a percentage of total cellular catecholamines. In each case values shown are means ± range of duplicates for a representative experiment. Similar results were obtained in two identical experiments performed on different cell preparations. Note that both control and PTX-treated cells exhibit similar $[Ba^{2+}]_o$ dependence. However, the extent of CA release in PTX-treated cells is greater by ca. 10% than in control cells. Also compare with CA release at zero [dihydropyridine]$_o$ (Fig. 1)

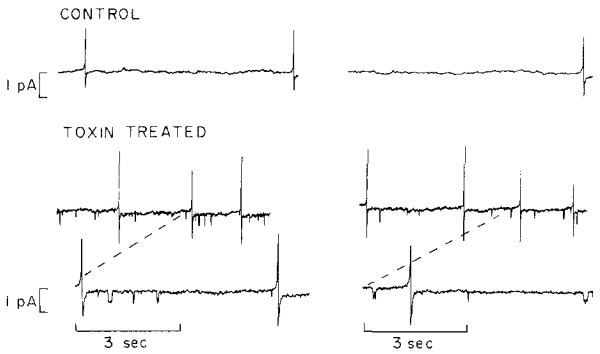

Fig. 5. Effect of pertussis toxin on spsontaneous electrical activity. *Control:* Two segments of a continuous record of spontaneous electrical activity from a control untreated chromaffin cell. *Toxin treated:* Four segments of a continuous record of spontaneous electrical activity from a cell pretreated with PTX for 5 hr from the same cell preparation. Time calibration is valid for both control and toxin-treated cells. Dashed lines indicate the initiation of the record on expanded time base shown below (calibration represents 0.75 sec). Pipette potential was set at 20 mV (estimated membrane potential −80 mV). The mean value for untreated cells with spontaneous electrical activity was 12.3 ± 4.9 min^{-1} (n = 5 cells). Including those chromaffin cells which did not exhibit spontaneous electrical activity, the mean value is 5.9 ± 2.6 min^{-1} (n = 12 cells)

high Ca^{2+} solution in the pipette (*see* Materials and Methods). The high Ca^{2+} solution was also used to record and to study the effects of PTX on Ca^{2+}-channel activity. For the present series of experiments the mean frequency of action potentials recorded from control cells was 12.3 ± 4.9 min^{-1}, and toxin treatment markedly increased the frequency of spontaneous action potentials to 44.7 ± 10.9 per min (Fig. 5, lower records).

Calcium Channels Activated by PTX

With high $[Ca^{2+}]$ in the pipette cell-attached patches on chromaffin cells exposed to PTX always showed channel events as brief downward deflections representing the flow of positive charge (presumably Ca^{2+}) across the patch from the solution in the pipette into the cell (Fig. 5, lower records). We then tested the channel for permeation by Mg^{2+} and Ba^{2+} on cell-attached membrane patches. With Mg^{2+} in place of Ca^{2+} in the pipette solution (in mM: 25 $MgCl_2$, 140 TMA-Cl, 10 Na-HEPES at pH 7.4) spontaneous action potentials were detected but no channel activity was apparent (*not shown*). In contrast, with Ba^{2+} in the pipette (in mM: 25 $BaCl_2$, 140 TMA-Cl, pH at 7.4), we observed not only the appearance of action potentials, but also brief channel openings. Thus, this new channel appears to be impermeant to Mg^{2+}, but highly permeant to Ba^{2+} (*not shown*). These results suggest that PTX treatment activated a new Ca^{2+}-selective channel.

A systematic analysis showed that all chromaffin cells pretreated with PTX (n = 20 cells) exhibited a significant increase in the frequency of Ca^{2+}-channel openings. Once activated, the channel remained active on inside-out excised patches exposed to the extracellular solution (2.6 mM $[Ca^{2+}]_o$). The frequency of channel openings was insensitive to the membrane potential across the patch. Changes in pipette potential in the range from −20 to 60 mV caused no measurable changes in the frequency of openings (Fig. 6*A*). The corresponding graph of the single Ca^{2+}-channel current as a function of pipette potential curve shown in Fig. 6*B* gave a slope conductance of 5.1 ± 0.3 pS. In contrast, untreated cells showed infrequent channel events. With 25 mM Ca^{2+} in the pipette the average amplitude was −0.42 ± 0.05 pA at a pipette potential of 0 mV (n = 5 cells).

Effects of PTX on Whole-Cell Calcium Currents

Figure 7 shows two sets of superimposed Ca^{2+} current records for a control cell (Fig. 7, left side) and for a cell which had been exposed to the toxin for

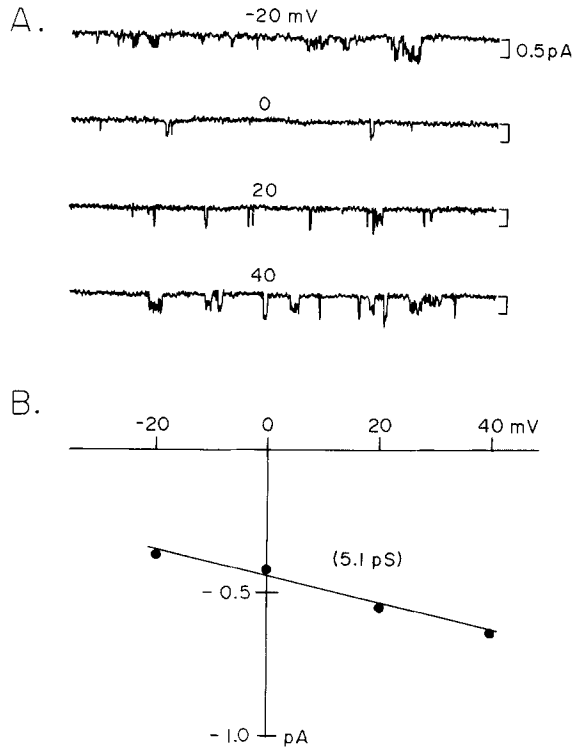

Fig. 6. Single Ca^{2+}-channel currents. (*A*) Segments of a continuous record of the currents across a cell-attached membrane patch from a chromaffin cell treated with PTX. Pipette potential (indicated above the single channel current records) was set at 20, 0, 20 and 40 mV with respect to the solution bathing the cells. Time elapsed between the segments shown varied from record to record and included periods with electrical activity. (*B*) Current-voltage relationship and single-channel conductance. Single Ca^{2+}-channel currents were measured from records digitized using a digital storage oscilloscope. They were filtered at 500 Hz. Extrapolated straight line cut the abscissa at −85 mV. The reversal potential can be used to estimate the selectivity ratio P_{Ca}/P_K as follows:

$$V_{rev} = RT/F\{\ln(4\ P^*_{Ca}[Ca^{2+}]/P_K[K^-])\}$$

and

$$P^*_{Ca} = P_{Ca}/\{1 + \exp[(V_{rev}F)/RT]$$

where R, T and F have their usual meanings. Taking the chromaffin cell resting potential as −65 mV, the reversal potential is estimated as 20 mV. Assuming $[K^+]_i$ as 120 mM, we estimate the permeability ratio as P_{Ca}/P_K of = 8.3

ca. 7 hr prior to the recording of the currents (Fig. 7, right side). Although the two sets of current records shown in Fig. 7 look rather similar, there are important differences both in activation kinetics and in the size of the current at each potential.

To compare the amplitude of the L-type Ca^{2+} currents in PTX-treated cells with that of control cells, we constructed current-voltage relationships using the results from one experiment in which the effects of PTX were maximal. As illustrated in Fig. 8, the size of $i_{Ca,max}$ for negative potentials was found to be always greater in the PTX-treated cell than in the control cell. This situation was reversed for positive potentials although the Ca^{2+} conductance, estimated as the slope of the current-voltage curve (points at 10, 20 and 30 mV), remained unchanged at 6.5 nS/cell. Assuming that single Ca^{2+}-channel conductance is not changed by PTX, the observation that the Ca^{2+} conductance remained unchanged at 6.5 nS/cell, suggest that PTX treatment does not cause the recruitment of inactive L-type Ca^{2+} channels.

Effect of PTX on the Steady-State and Kinetic Characteristics of the Noninactivating L-type Calcium Channel

To determine the effects of PTX on steady-state and kinetic properties of voltage-gated Ca^{2+} channels we used a modified version of the Hodgkin-Huxley model (Ceña et al., 1989). The individual *I-V* curves were used to obtain m_∞ curves for both control and treated cells at various potentials from −40 to 50 mV. The m_∞ curve from a typical PTX-treated cell (■) is plotted together with the activation curve from control cells (□) in Fig. 9. While the effective valence z_e of the voltage sensor of the L-type Ca^{2+} channel remained unchanged at −2.5 electronic charges, the midpoint potential V_0 was shifted from −3.6 mV (n = 12) in control cells (□) to −16.4 mV in a PTX-treated cell (■). The average value for V_0 was −11.4 ± 3.8 mV (n = 6).

For kinetic analysis of the currents, we fitted the empirical Eq. (1) to the records.This procedure generated values for the activation time constants. Shown in Fig. 9 (lower part) are the mean values of the activation time constant τ_m in control cells (n = 11, □) and treated cells (n = 6, ■). For pulses taking the membrane potential from the holding level of −80 mV towards positive values, τ_m was always smaller for the PTX-treated cells. The meaning of this result is that the size of the activation rate constant α is greater in the toxin-treated cells. The effects on the rate constant for deactivation of the L-type Ca^{2+} channel were less obvious. Resolving Eqs. (3*a*) and (3*b*) for $\alpha(V)$ and $\beta(V)$ allowed us to verify the effect of PTX on the individual rate constants (*see* Table).

To compare the effects of various activators and/or modulators of CA secretion we constructed the Table in which we have taken the parameters obtained from the Boltzmann distribution analysis as presented in Fig. 9 and in a previous publication

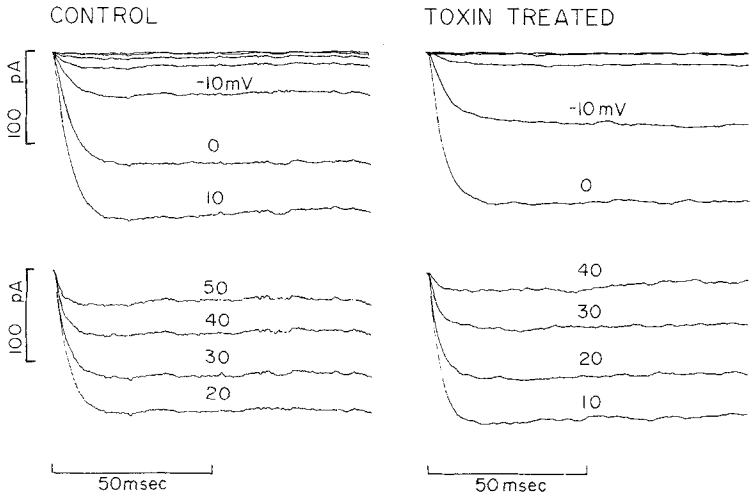

Fig. 7. Effect of pertussis toxin on L-type calcium currents. *Left side:* Superimposed inward Ca^{2+}-current records from a control cell. *Right side:* Whole Ca^{2+} currents from a cell treated with PTX. Holding potential was set at −80 mV for both cells. Membrane potential during the depolarizing pulses was changed in 10-mV steps

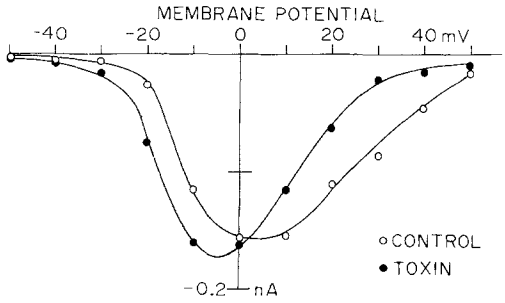

Fig. 8. Current-voltage relationships for control and toxin-treated cells. Maximum inward Ca^{2+} currents for control (○) and PTX-treated cells (●). Holding potential was set at −80 mV

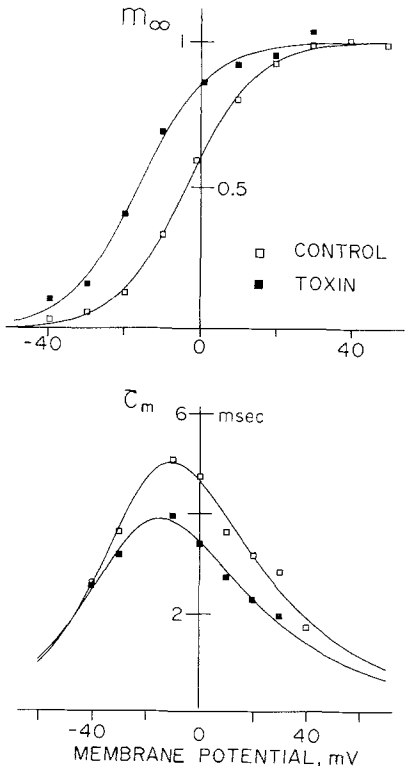

Fig. 9. Effect of pertussis toxin on steady-state activation and on time constant for the L-type Ca^{2+} current. *Upper panel:* Normalized mean m_∞ values in control cells (n = 12, ○) and in a toxin treated cell (●). The solid line represents the best fit to Eq. (2). Fitted curves were calculated with the following parameters: ○: a = −2.5, V_0 = −3.6 mV (control); ●: a = −2.5, V_0 = −16.4 mV (toxin-treated cells). *Lower panel:* Time constant, τ_m, for activation of the Ca^{2+} current in control chromaffin cells (○; n = 6) and in a typical cell after preincubation in the presence of PTX (100 nM) as a function of membrane potential. Fitted curves were calculated with Eqs. (3) and (4). The following parameters were used for the best fit: ○: A = 139.7 sec^{-1}, a = 0.67, V_0 = −7.9 mV, B = 55.7 sec^{-1}, b = −1.37 and V_0 = −7.9 mV (control cells). ●: A = 159.0 sec^{-1}, a = 0.67, V_0 = −15.2, B = 91.6 sec^{-1}, b = −1.24, V_0 = −15.2 mV (toxin-treated cells)

from our laboratory (Ceña et al., 1989). The effective charge on the L-type Ca^{2+}-channel gate is clearly augmented by increasing $[Ca^{2+}]_0$ from the physiological level of 2.6 to 5.2 mM and by the Ca^{2+}-channel agonist Bay K-8644. In contrast, pertussis toxin does not affect the effective valence of the voltage sensor of the Ca^{2+} channel. Therefore, the three treatments, namely high $[Ca^{2+}]_0$, Bay K-8644 and PTX, induce a substantial shift of the midpoint potential V_0 towards more negative values. The shift induced by high $[Ca^{2+}]_0$ is explained by assuming that ψ_i remains unchanged and that Ca^{2+} induces a reversal of the surface charge (Ceña et al., 1989). Unlike the effect of high $[Ca^{2+}]_0$ on the outer membrane surface charge, Bay K-8644 appears to increase the charge density of the inner side of the membrane.

Discussion

The mechanism by which pertussis toxin acts to promote or potentiate exocytosis in chromaffin cells appears to be based on the toxin's ability to alter the

Table. Effects of various potentiators of CA secretion on parameters of the empirical functions used to describe L-type Ca^{2+}-channel gating

	2.5 Ca^{2+}	5.2 Ca^{2+} [a]	Bay K-8644[a]	PTX
z_e[e.c.]	−2.5	−3.2	−3.6	−2.5
V_o[mV]	−3.6	−11.2	−12.9	−16.4
kT/z_e	9.8	7.8	7.0	9.8
A[sec^{-1}]	139.7	144.4	95.0	159.0
a[e.c.]	−0.67	−0.57	−0.72	−0.67
B[sec^{-1}]	55.7	47.3	38.0	91.6
b[e.c.]	−1.37	−1.25	−1.0	−1.24
V_o[mV]	−7.9	−9.9	−14.8	−15.2

z_e: Effective valence in electronic charges (e.c.); [Bay K-8644] = 100 nM; [PTX] = 100 ng/ml.
[a] From Ceña et al., 1989.

activity of at least two types of Ca^{2+} channels. One of these channels is the L-type Ca^{2+} channel, in which the open-state probability curve is shifted and the channel kinetics is profoundly modified. Specifically, PTX treatment causes the mid-point potential, V_o, to be shifted to a more negative value. PTX also substantially elevates the rate constant for activation of the L-type Ca^{2+} channel. The functional consequences of these changes are that Ca^{2+} entry is faster and is more likely to occur at potentials near the resting membrane potential. Thus, a slight depolarization of the membrane, which under control conditions would have little effect on L-type Ca^{2+}-channel function, would now substantially increase inward Ca^{2+} current. We presume, based on the available data, that such a process might explain the potentiating effects of PTX on secretion induced by physiological secretagogues, as well as play a role in the intrinsic induction of CA secretion by PTX alone.

However, pertussis toxin-activated secretion is only partially inhibited by pharmacologically relevant doses of dihydropyridine drugs. In different experiments, niludipine and nifedipine inhibited PTX-induced secretion by 30–59% and 51–59%, respectively. To us, these data indicate that other Ca^{2+} pathways might also be involved in PTX action. This second process may involve the G-type calcium channel also reported here, inasmuch as it is only found in PTX-treated cells and is insensitive to the dihydropyridine drugs. G-type Ca^{2+} channels are voltage insensitive, Ca^{2+}-selective channels with relatively low conductance (ca. 5 pS). This type of channel was first detected in our own laboratory (Rojas et al., 1990), in glucose-activated human pancreatic β-cells, and for that reason we called "G-type" (G for glucose). It is possible that PTX treatment opens these channels, slightly depolarizes the cells, and thereby recruits channel activity by PTX-modified L-type Ca^{2+} channels.

The low conductance of this G-type Ca^{2+} channel is consistent with the fact that we were unable to detect an appreciable difference in the macroscopic whole cell Ca^{2+} conductance (ca. 6.5 nS/cell) of PTX-treated or control cells when compared in the whole cell recording mode. This similarity is also due to the fact the conductance of the L-type Ca^{2+} is not intrinsically modified; only the activation curve is shifted towards more negative membrane potentials. Furthermore, that the slow time course of PTX-induced secretion indicates that in any event one ought not necessarily expect abrupt and substantial increases in Ca^{2+} current. These data are also consistent with reports that PTX-potentiated secretion from chromaffin cells is not accompanied by potentiation of $^{45}Ca^{2+}$ uptake (Tanaka et al., 1987), or increases in $[Ca^{2+}]_i$ (Sasakawa et al., 1988).

Pertussis toxin is believed to act on cell processes by inactivating a G-protein (for review *see* Bourne, 1988; Rosenthal et al., 1988). In the chromaffin cell G-proteins are distributed on both plasma membranes and chromaffin granule membrane (Toutant et al., 1987; Burgoyne & Morgan, 1989), and PTX-mediated catecholamine secretion is induced in proportion to the extent of ADP-ribosylation of a 40-kD protein(s) (Brocklehurst & Pollard, 1988). Thus, it is possible that the multiple actions of PTX on Ca^{2+} channels in chromaffin cells is mediated by inactivation of one or several inhibitory G-proteins. In the case of the L-type Ca^{2+} channel, we know of no previous reports of direct G-protein dependent interactions. Indeed, the kinetic analysis of the PTX-treated cells shows that the toxin actually causes a shift in the midpoint potential V_o for the activation curve from −3.6 to −16.4 mV. Since V_o represents the difference between the surface potential of the inner aspect of the membrane (ψ_i) minus the surface potential of the outer aspect of the membrane (ψ_o), the meaning of this result is that the charge near the internal side of the L-type Ca^{2+} channel is increased in PTX-treated cells. A possible explanation for this effect is that PTX may promote phosphorylation of the channel.

Of course, the concept of controlling the L-type Ca^{2+} channel by changes in the degree of phosphorylation is not new. We (Ceña et al., 1989) and others (Chad, Kalman & Armstrong, 1987) have proposed that the Ca^{2+}-channel agonist Bay K-8644 stabilizes the phosphorylated conformation of the channel, and that this process is responsible for activation by Bay K-8644. However, data in the present paper show that PTX and Bay K-8644 act by entirely different mechansims. In the case of Bay K-8644, the drug decreases the rate constant for turning *off* the Ca^{2+}

current (Ceña et al., 1989). In contrast, PTX increases the rate constant for turning *on* the Ca^{2+} current and also changes V_0. Thus, Bay K-8644 and PTX have similar consequences as *potentiators* of secretion, although the mechanisms are quite different. More profoundly, PTX itself promotes secretion, while Bay K-8644 does not. It thus follows that if phosphorylation is the mechanism by which PTX exerts its effects on the L-type Ca^{2+} channel, it does so at a distinct site(s) from that utilized by Bay K-8644.

The mechanism by which PTX activates the G-type Ca^{2+} channel is unclear. Since the G-type channel can only be detected after PTX treatment, we have no basis for a mechanistic comparison with the native channel. However, for the G-type channel, as for the L-type channel, the meaning of activation following PTX treatment is that both types of channels are in a *tonically inhibited* state in the control cell. In the case of the G-type channel, the channel is closed; in the case of the L-type channel, the channel is less prone to open in the polarized state of the control cells. These findings stand in sharp contrast to the situation in neutrophils (Gomperts, Barrowman & Crockcroft, 1986) and mast cells (Cockcroft et al., 1987), in which PTX partially *inhibits* exocytosis. In the latter cells, it has been hypothesized that a unique G-protein exists to promote secretion, termed "G_{EX}," which must be *tonically* activated. It is worth noting that the concept of potentiation of secretion by PTX is not unique to chromaffin cells. In the case of β-cells in the islet of Langerhans, Islet Activating Protein (IAP) is known to be pertussis toxin (Sekura, Moss & Vaughan, 1985).

References

Bourne, H.R. 1988. Preliminary structural model of the G-protein alpha-chain. *Adv. Second Messenger Phosphorylation Res.* **21:**33–38

Brocklehurst, K., Pollard, H.B. 1988. Pertussis toxin stimulates delayed-onset, Ca-dependent catecholamine release and the ADP ribosylation of a 40 kDa protein in bovine chromaffin cells. *FEBS Lett.* **234:**439–445

Brocklehurst, K.W., Morita, K., Pollard, H.B. 1985. Characterization of protein kinase C and its role in catecholamine secretion from bovine adrenal-medullary cells. *Bichem. J.* **228:**35–42

Brown, A. M., Birnbaumer, L. 1988. Direct G-protein gating of channels. *Am. J. Physiol.* **254:**H401–H410

Burgoyne, R., Morgan, A. 1989. Low molecular mass GTP-binding proteins of adrenal chromaffin cells are present on the secretory granule. *FEBS Lett.* **245:**122–126

Ceña, V., Stutzin, A., Rojas, E. 1989. Effects of calcium and Bay K-8644 on calcium currents in adrenal medullary chromaffin cells. *J. Membrane Biol.* **112:**255–265

Chad, J., Kalman, D., Armstrong, D. 1987. The role of cyclic AMP-dependent phosphorylation in the maintenance and modulation of voltage activated calcium channels. *Soc. Gen. Physiol. Ser.* **42:**167–186

Cockcroft, S., Howell, T.W., Gomperts, B. 1987. Two G-proteins act in series to control stimulus-secretion coupling in mast cells, use of neomycin to distinguish between G-proteins controlling phosphoinositide phosphodiesterase and exocytosis. *J. Cell Biol.* **205:**2745–2750

Forsberg, E.J., Pollard, H.B. 1988. Ba^{2+}-induced ATP release from adrenal medullary chromaffin cells is mediated by Ba^{2+} entry through both voltage and receptor gated Ca^{2+} channels. *Neuroscience* **27:**711–715

Goldman, D.E. 1943. Potential, impedance and rectification in membranes. *J. Gen. Physiol.* **27:**37–60

Greenberg, A., Zinder, O. 1982. Catecholamine secretion from isolated adrenal medulla cells. *Cell Tissue Res.* **226:**655–665

Gomperts, B.D., Barrowman, M.M., Cockcroft, S. 1986. Dual role for guanine nucleotides in stimulus-secretion coupling. *Fed. Proc.* **47(7):**2156–2161

Heldman, E., Levine, M., Raveh, L., Pollard, H.B. 1989. Barium ions enter chromaffin cells via voltage-dependent calcium channels and induce secretion by a mechanism independent of calcium. *J. Biol. Chem.* **264(14):**7914–7920

Hodgkin, A.L., Katz, B. 1949. The effect of sodium ion on the electrical activity of the giant axon of the squid. *J. Physiol.* **108:**37–77

Neher, E., Almers, W. 1986. Patch pipettes used for loading small cells with fluorescent indicators. *Adv. Exp. Med. Biol.* **211:**1–5

Pollard, H.B., Ornberg, R., Levine, M., Heldman, E., Morita, K., Kelner, K., Lelkes, P.I., Brocklehurst, K., Forsberg, E., Duong, L., Levine, R., Youdim, M.B.H. 1985. Hormone secretion by exocytosis with emphasis on information from the chromaffin cell system. *Vitam. Horm.* **42:**109–196

Rojas, E., Hidalgo, J., Carroll, P., Li, M.X., Atwater, I. 1990. A new class of calcium channels activated by glucose in human pancreatic beta cells. *FEBS Lett.* **261:**265–270

Rosenthal, W., Hescheler, L., Trautwein, W., Schultz, G. 1988. Receptors and G-protein mediated modulation of voltage-dependent calcium channel. *FASEB J.* **2:**2784–2790

Sasakawa, N., Yamamoto, S., Nakaki, T., Kato, R. 1988. Effects of islet-activating protein on the catecholamine release, Ca^{2+} mobilization and inositol triphosphate formation in cultured adrenal chromaffin cells. *Biochem. Pharmacol.* **37:**2485–2487

Sekura, R.D., Moss, J., Vaughan, M. 1985. Pertussis Toxin. Academic, Orlando, FL

Stutzin, A., Stojilkovic, S., Catt, K.J., Rojas, E. 1989. Characteristics of two types of calcium channels in rat pituitary gonadotrophs. *Am. J. Physiol.* **257:**C865–C874

Tanaka, T., Yokohama, H., Negishi, M., Hayashi, H., Ito, S., Hayashi, O. 1987. Pertussis toxin facilitates secretagogue-induced release from cultured bovine adrenal chromaffin cells. *Biochem. Biophys. Res. Commun.* **144:**907–914

Toutant, M., Aunis, D., Bockaert, J., Homburger, V., Rouot, B. 1987. Presence of three pertussis toxin substrates and G_o^α immunoreactivity in both plasma and granule membranes of chromaffin cells. *FEBS Lett.* **215:**339–344

Received 13 September 1990; revised 2 January 1991