

# Long-term potentiation of nicotinic synaptic transmission in rat superior cervical ganglia produced by phorbol ester and tetanic stimulation

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## Abstract

The long-term potentiation of nicotinic synaptic transmission induced by both active phorbol ester (4 $\beta$ -phorbol-12,13-dibutyrate, PdBu) and tetanic trains of preganglionic stimulation was studied in single neurons of the superior cervical ganglion (SCG) of the rat using intracellular recording techniques. PdBu significantly increased the mean amplitude of both the unitary evoked fast excitatory postsynaptic potentials (EPSPs) and the fast excitatory postsynaptic currents (EPSCs) to  $17.0 \pm 3.3$  mV (control  $8.4 \pm 1.9$  mV,  $n=5$ ) and  $2.8 \pm 0.4$  nA (control  $0.8 \pm 0.1$  nA,  $n=10$ ), respectively. There was no significant change in either the resting membrane potential, input resistance, or the threshold for the initiation of an action potential. The response to exogenously applied acetylcholine (ACh) was also not changed following exposure to PdBu. In low-calcium, high-magnesium solutions, PdBu significantly increased the quantal content of EPSPs approximately threefold from a control of  $0.9 \pm 0.2$  ( $n=5$ ) to  $2.6 \pm 0.6$  ( $n=5$ ). The quantal content of EPSCs was also increased to  $1.3 \pm 0.2$  (control  $0.5 \pm 0.1$ ,  $n=10$ ). PdBu increased the frequency of miniature EPSPs (mEPSPs) to  $196 \pm 47\%$  ( $n=6$ ) of control, while the amplitude, rise time, rate of rise, and decay of mEPSPs were not significantly changed. Tetanic stimulation significantly increased the amplitude of the unitary synaptic EPSPs and EPSCs without significantly changing the resting membrane potential, input resistance, threshold for initiation of an action potential, or the response to exogenously applied ACh. Tetanic stimulation significantly increased quantal content of EPSPs and EPSCs threefold. The results obtained with tetanically induced LTP are similar to the results obtained with phorbol ester-induced LTP in these ganglion neurons. These results suggest that both tetanically induced and phorbol ester-induced LTP, in the rat, share similar mechanisms which involve, at least in part, activation of PKC-dependent mechanisms to increase quantal release from sympathetic preganglionic axon terminals.

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**Keywords:** Tetanically induced LTP; Sympathetic preganglionic and postganglionic neurons; Autonomic neurons; Phorbol ester; Long-term potentiation; Protein kinase C; Synaptic modulation; Rat SCG; Autonomic ganglia; Intracellular mediators; Second messengers; Cell signaling

## 1. Introduction

Enhancement of synaptic transmission that lasts for minutes, hours, or days is found in central as well as peripheral neuronal synapses in response to brief trains of tetanic stimulation (Dunant and Dolivo, 1968; Kuba and Kumamoto, 1990). Since the time course of this enhancement is frequently long-lasting, the induction and maintenance of long-term potentiation (LTP) has been thought to be associated with the activation of intracellular second

messenger systems at either a presynaptic or postsynaptic site (Linden and Routtenberg, 1989; Kuba and Kumamoto, 1990; Briggs, 1995). Protein kinase C (PKC) is a Ca<sup>2+</sup>/phospholipid-dependent protein kinase that has been shown to play an important role in regulating synaptic efficacy (Briggs, 1995; Kaczmarek, 1987; Bachoo et al., 1992). The involvement of PKC in LTP is supported by the finding that these protein kinases (i) are found in high concentrations in the nervous system (Nishizuka et al., 1984; Kikkawa and Nishizuka, 1986; Castagna et al., 1982; Kikkawa et al., 1982; Nishizuka, 1986); (ii) significantly enhance neurotransmitter release (Pozzan et al., 1984; Publicover, 1985; Zurgil and Zisapel, 1985; Eusebi et al., 1986; Guitart et al., 1990); and (iii) lead to a potentiation of synaptic transmission in the hippocampus (Malinow et al., 1988; Malenka et al., 1989; Linden and Routtenberg, 1989;

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Colley et al., 1990; Matthies et al., 1991). The degree of hippocampal LTP is correlated to the amount of PKC translocated from the cytosol to the cell membrane (Akers and Routtenberg, 1987), supporting the finding that active phorbol esters and LTP in the hippocampus share, in part, similar underlying mechanisms (Bliss and Lomo, 1972; Malenka et al., 1989; Lovinger and Routtenberg, 1988; Reymann et al., 1988).

Previous studies have shown that, in sympathetic ganglia, synaptic activity is coupled to an increase in phosphoinositol hydrolysis (Briggs et al., 1985b; Linden and Routtenberg, 1989) and that protein kinase C-activating phorbol esters, but not inactive phorbol esters, produce a long-lasting potentiation of the postganglionic compound action potential (CAP) evoked by preganglionic stimulation in the perfused rat superior cervical ganglion (SCG) (Bachoo et al., 1992). At the same synapse, the synaptically evoked LTP, which previously was shown to involve a presynaptic mechanism in the rat (Briggs et al., 1985a; Briggs and McAfee, 1988; Briggs, 1995), was occluded by the phorbol ester-induced potentiation and was attenuated by inhibitors of PKC (Bachoo et al., 1992). Furthermore, pretreatment of the rat SCG with active phorbol esters markedly reduced the potentiation of nicotinic transmission induced by either a subsequent application of active phorbol esters or tetanic stimulation. These findings suggest that the activity-induced and phorbol ester-induced LTP may share a common mechanism (Bachoo et al., 1992). However, in these previous studies using active phorbol esters, extracellular recording techniques were used and the site(s) of action for the induced LTP could not be determined. In the present experiments, we used intracellular recording techniques to examine the contributions of presynaptic vs. postsynaptic sites to LTP induced by PdBu, and compared the results to those obtained with tetanic stimulation under the same experimental conditions. Intracellular recordings were performed in the acutely excised rat SCG. Synaptic evoked excitatory postsynaptic potentials (EPSPs) and excitatory postsynaptic currents (EPSCs) evoked by preganglionic stimulation, and postsynaptic potentials evoked by the application of exogenous acetylcholine (ACh) were recorded along with spontaneous mEPSPs. The results suggest that in the rat SCG, the potentiation of nicotinic ganglionic transmission induced by PdBu and tetanic stimulation is the result of an increase in neurotransmitter release, caused primarily by an increase in the number of quanta released.

## 2. Materials and methods

### 2.1. Dissection and mounting

Sprague–Dawley rats (200–300 g) were euthanized with sodium pentobarbital (35 mg/kg ip) according to a

research protocol approved by the local Institutional Animal Care and Use Committee at the University of Vermont. The SCG, along with the cervical sympathetic trunk (CST) and the postganglionic nerves, was removed and transferred to a Sylgard-covered plastic chamber (1.0 ml volume) perfused at the rate of 1.0 ml/min with a solution containing (mM): NaCl, 130; KCl, 4.0; MgCl<sub>2</sub>, 1.0; KH<sub>2</sub>PO<sub>4</sub>, 1.0; NaHCO<sub>3</sub>, 12.0; CaCl<sub>2</sub>, 2.0; glucose, 11.0. The solution was equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4) and contained atropine sulfate (2 μM). All experiments were performed at 22 °C. To facilitate the impalement with microelectrodes, the connective tissue sheath was carefully split longitudinally, gently spread apart, and pinned to the chamber bottom. 4β-phorbol-12,13-dibutyrate (PdBu) was added to the perfusion solution and applied to the ganglion for 5 min.

### 2.2. Electrical recordings

Ganglion neurons were impaled with glass microelectrodes filled with 3 M KCl or 2 M K acetate (50–120 MΩ resistance). A high-impedance bridge amplifier (Axoclamp 2A; Axon Instruments, Foster City, CA, USA) was used for recording in the current- or voltage-clamp mode. The membrane potential was measured as the potential difference between the intracellular microelectrode and a reference electrode placed in the bath. The input resistance was determined by injecting constant current hyperpolarizing pulses (0.1–0.3 nA, 100–300 ms) through the microelectrode. The threshold for action potential generation was measured as the membrane potential at which a single action potential was evoked in approximately 50% of the trials by depolarizing current pulses (100–300 ms) of progressively increasing amplitude. EPSPs or EPSCs were evoked by single square wave stimuli (0.5 ms) from a Grass S48 stimulator, applied to the CST with a suction electrode.

The membrane potential was held constant with current injection, except in the experiments in which the effects of PdBu- or tetanically-induced LTP were examined on the resting membrane potential, input resistance, and the threshold for action potential generation. LTP was induced in separate experiments either by application of PdBu to the ganglion or by stimulation of the CST with a single, supramaximal tetanic train (20 Hz, 20 s). LTP is defined as a significant increase in the evoked synaptic response that is maintained for a minimum of 20 min. In our experiments, the duration of LTP was at least 1 h. Posttetanic potentiation in the rat SCG decays to an insignificant level in 5–10 min (Brown and McAfee, 1982, 1988; Zengel et al., 1980); therefore, intracellular recordings were resumed 20 min either following tetanic stimulation or after the addition of PdBu. Previous studies from our laboratory (Bachoo et al., 1992) examined the concentration–response relationship for PdBu on the time course of the LTP using extracellular CAP recordings. We

established that potentiation of the CAP occurred with concentrations of active phorbol esters of 1 nM with maximum responses reached at 1  $\mu$ M PdBu (Bachoo et al., 1992). A concentration of 1  $\mu$ M was, therefore, chosen for these experiments.

Quantal content and quantal size of EPSPs or EPSCs were measured in neurons in which the synaptic input was reduced to a single fast excitatory input (Kuno, 1971; Sacchi and Perri, 1971, 1973; McLachlan, 1975). In several neurons in which release was very low, quantal content ( $m$ ) was calculated from the ratio of the number of stimuli applied to the CST to the number of stimuli that failed to evoke a postsynaptic response:  $m = \ln(\text{number of stimuli})/\text{number of failures}$ . In most neurons, quantal content was calculated as the ratio of the mean amplitude of EPSPs (EPSCs) to the mean amplitude of mEPSPs (mEPSCs) (Del Castillo and Katz, 1954). The mEPSPs recorded before and after tetanic stimulation or phorbol ester application were used to calculate the mean and variance of the quantal size. In addition, the ratio of the mean EPSC or EPSP amplitude to quantal content for each neuron was used to calculate the quantal size ( $q$ ). To obtain unitary event recordings with supramaximal stimulation, the  $\text{CaCl}_2$  concentration in the control solution was decreased to 0.5 mM and the  $\text{MgCl}_2$  concentration was increased to 2–10 mM. Each experiment consisted of an initial recording period to establish a stable resting membrane potential followed by a period during which constant current pulses were delivered to the neuron to determine the input resistance. Following either tetanic stimulation or application of PdBu, test stimuli to the CST were applied at a rate of one per minute to assess the duration of the synaptic potentiation.

Neurons were maintained at membrane potentials ranging from  $-60$  to  $-120$  mV to reduce the generation of action potentials. Most neurons were maintained at  $-100$  mV and at this potential had unitary EPSP amplitudes ranging from 2.5 to 10 mV. The EPSP–EPSC data were digitized, stored, and analyzed with a Micro PDP/11 laboratory computer (Digital Equipment, Maynard, MA, USA), using an Indec Chesire Interface (Indec Systems, Sunnyvale, CA, USA). The SCAN software program (Strathclyde Electrophysiology Software; University of Strathclyde, Glasgow, Scotland, UK) was used for the acquisition and analysis of mEPSPs.

The resting frequency of spontaneous mEPSPs in some autonomic ganglion neurons is very low under control conditions (Blackman et al., 1962; Blackman and Purves, 1969; Dennis et al., 1971). In some neurons, the probability of recording mEPSPs was increased by changing the KCl concentration in the superfusing solution from 4 to 20 mM (NaCl reduced accordingly). In each experiment, the mEPSPs were recorded under the same experimental conditions during the control period and following PdBu or tetanic stimulation. The mean amplitudes of mEPSPs were expressed as a percent of control. The mEPSPs

recorded in a neuron after tetanic stimulation or after PdBu were increased in frequency (Blackman and Purves, 1969; McLachlan, 1975) and were selected according to the requirement that the waveform started from and returned to baseline without artifacts or overlaps of other events. mEPSPs were averaged to obtain a mean trace by aligning individual events on the last sample point before the rise from baseline levels. The analysis of mEPSPs included the rise time, rate of rise, amplitude, and the decay time course. In some experiments, the spontaneous mEPSP amplitude analysis was performed in the presence of TTX to ensure that stimulation of axon collaterals or other presynaptic inputs by PdBu or tetanic stimulation did not alter this estimate. Large or multi-quantal responses, which represented fewer than 1% of the mEPSPs observed during this study, were excluded from the analysis.

To measure the responsiveness of the postsynaptic neuronal membrane to ACh, the tip of a pressure-perfused micropipette (50–100  $\mu$ m diameter), filled with ACh (0.1 M), was placed near the impaled neuron. ACh was ejected by a pressure application of 3 psi (0.2–0.5 s duration). Successive applications of ACh to single neurons were spaced at 5-min intervals.

Results are expressed as the mean  $\pm$  S.E.M. Differences between means were tested for significance using the paired or unpaired  $t$  test with  $P \leq 0.05$  considered statistically significant.

Atropine and 4 $\beta$ -phorbol-12,13-dibutyrate (PdBu) were obtained from Sigma (St. Louis, MO, USA).

### 3. Results

#### 3.1. Enhancement of synaptic nicotinic transmission after exposure to PdBu

Following exposure of ganglia to PdBu (5-min exposure), EPSP amplitude started to increase within 2 min and reached a peak between 10 and 40 min (Fig. 1). The amplitude of the EPSPs gradually recovered to control values in 90–100 min. The amplitude of the EPSP increased significantly to  $17.0 \pm 3.3$  mV ( $n=5$ ) from an initial value of  $8.4 \pm 1.9$  mV ( $n=5$ ,  $P < 0.05$ ) when recorded 20 min after PdBu exposure (Fig. 1). In a number of experiments, the evaluation of the amplitude of EPSPs could not be made because 80–100% of the potentiated EPSPs generated an action potential (Fig. 1). When neurons were voltage-clamped, the amplitude of the EPSC was also significantly increased (Fig. 2A–C). Fig. 2D illustrates a histogram of EPSC amplitudes generated from a single neuron both before and following PdBu. The mean amplitude of the control unitary EPSC was  $0.8 \pm 0.1$  nA. Following PdBu, the mean EPSC amplitude increased significantly to  $2.8 \pm 0.4$  nA ( $n=10$ ,  $P < 0.05$ ) (Fig. 2D).

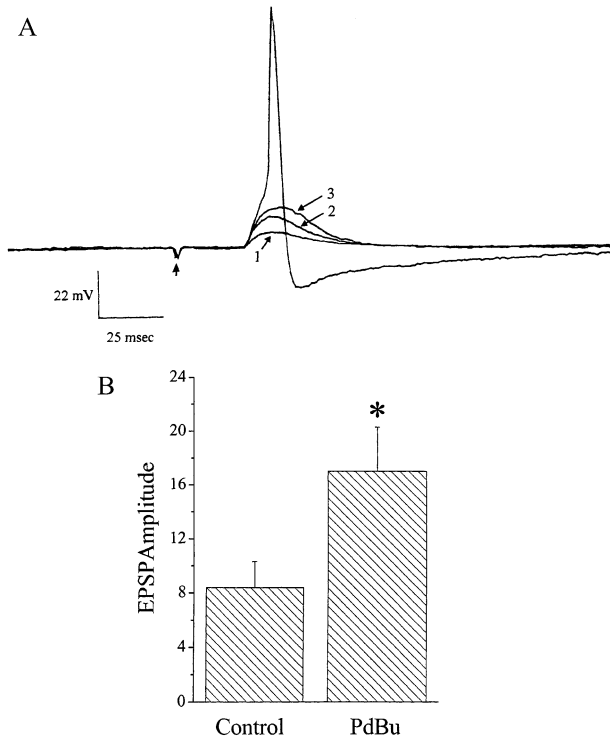


Fig. 1. PdBu increases the amplitude of the EPSP. (A) Consecutive superimposed EPSPs recorded from a neuron current-clamped at  $-70$  mV. Supramaximal preganglionic stimulation at one per minute throughout (stimulus artifact indicated by an arrow). In this neuron, the stimulus produced a subthreshold EPSP. The control EPSP in the figure is trace 1. After PdBu ( $1 \mu\text{M}$ ), the amplitude of the EPSP grew with successive preganglionic stimuli (traces 2 and 3) until it became suprathreshold. Calibration bars are 22 mV and 25 ms. (B) Summary of EPSP amplitude in control recordings and after PdBu.

### 3.2. PdBu did not alter the postsynaptic response to ACh

To test for a change in postsynaptic nicotinic receptor responsiveness following exposure to PdBu, ACh was applied at 5-min intervals both before and after PdBu with a pressure-perfusion micropipette in the presence of  $2 \mu\text{M}$  atropine. ACh produced a depolarization with a fast time to peak and a slow decay, lasting 5–10 s (Fig. 3A, control). Under control conditions, the peak amplitude of three consecutive ACh responses differed by less than 10%. To ensure that the response to ACh under control conditions was submaximal, ACh was applied in four steps of increasing duration, from 200 to 500 ms (Fig. 3A and B). This test was performed both before and during the first 40 min after exposure to PdBu. The responses to ACh before and after PdBu were not significantly different. Fig. 3C summarizes the results obtained in five neurons in which both the ACh response and the evoked EPSP were recorded from the same neuron both before and at various times after exposure to PdBu. There was no change in the amplitude of the depolarization produced by ACh applications during the potentiation of the EPSP. There was also no change in either

the value of the membrane potential, input resistance, or the threshold for initiation of a single action potential following PdBu in neurons in which the membrane potential was not controlled by current injection. The membrane potential following PdBu was  $-48.9 \pm 1.9$  mV,  $n=8$  (control,  $-49.1 \pm 1.3$  mV,  $n=10$ ,  $P > 0.05$ ). Input resistance values following PdBu were  $85 \pm 4 \text{ M}\Omega$ ,  $n=5$  (control,  $81.9 \pm 12.9 \text{ M}\Omega$ ,  $n=7$ ,  $P > 0.05$ ). The threshold for initiation of the

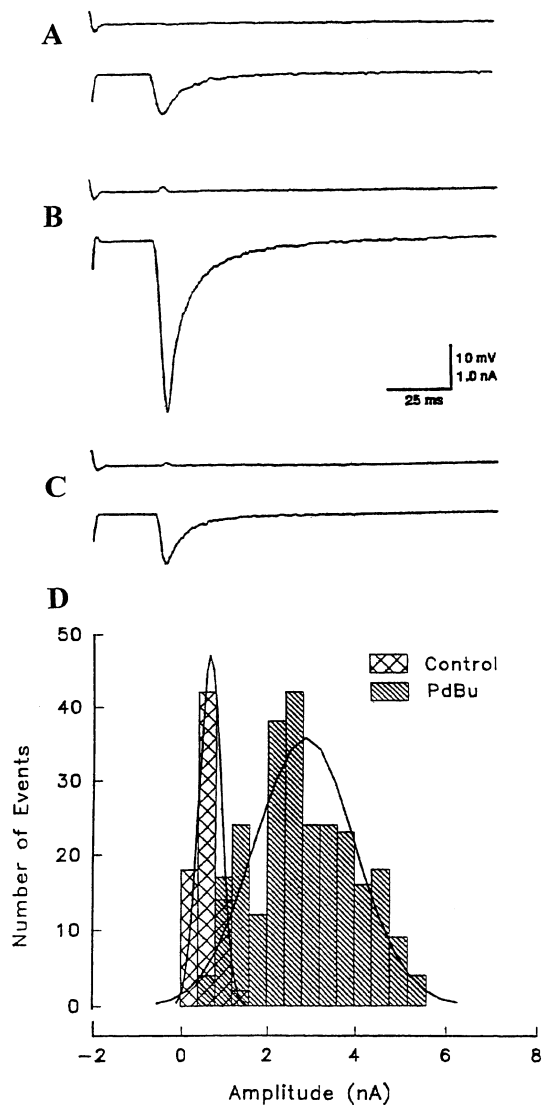


Fig. 2. PdBu increases the amplitude of the EPSC. Intracellular recording of the EPSC evoked by a preganglionic stimulus in a ganglion neuron voltage-clamped at  $-70$  mV under control conditions (A), 20 min (B) and 70 min (C) after the addition of PdBu. The voltage and current records are shown in the upper and lower traces of each panel, respectively. Each trace is the average of at least 25 responses. A marked, reversible increase in EPSC amplitude followed the addition of PdBu. The amplitudes were 0.7 nA (A), 2.8 nA (B), and 0.8 nA (C). (D) represents amplitude histograms of EPSCs recorded in control solution and 20 min after exposure to PdBu. Each set of data was fitted with a single Gaussian curve. Notice the significant increase in EPSC amplitude: mean EPSC values for this neuron were  $0.7 \pm 1$  nA in control solution and  $2.8 \pm 0.1$  nA following exposure to PdBu.

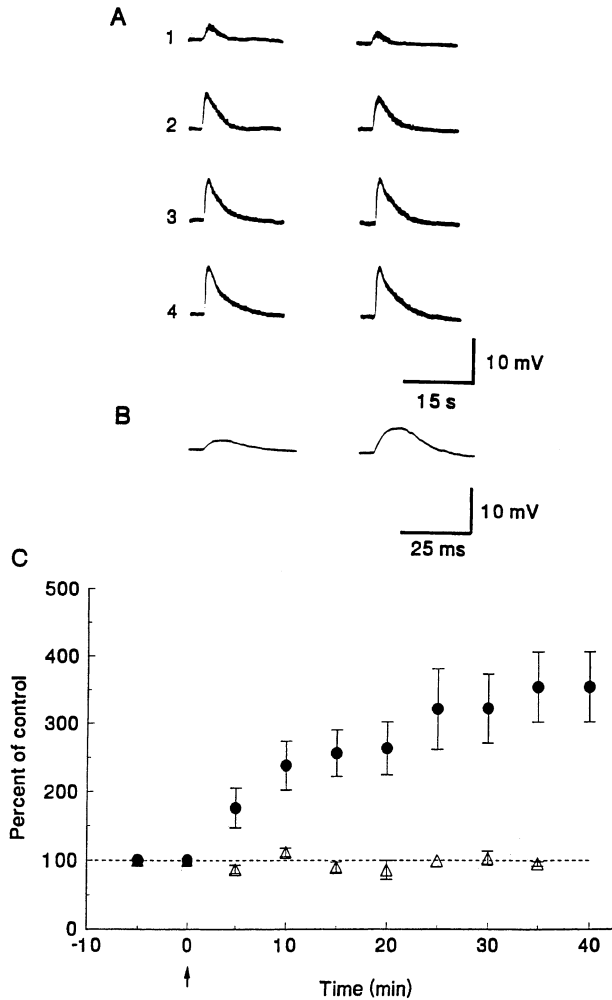


Fig. 3. Effects of PdBu on the amplitude of EPSPs and ACh-induced potentials. ACh-induced potentials (A) and EPSPs (B) were recorded from the same ganglion neuron in control solution and after the addition of PdBu (15 min). ACh puffs of increasing duration (trace 1, 200 ms; trace 2, 300 ms; trace 3, 400 ms; trace 4, 500 ms) were applied to the same neuron. Averaged evoked EPSPs are shown in (B). Holding potential was  $-60$  mV. (C) shows the action of PdBu on the mean EPSP amplitude (closed circles) and on the mean ACh-induced potential (open triangles) ( $n=5$ ). PdBu was added at the arrow. Values are expressed as a percent of control. Vertical bars indicate the S.E.M. Notice the marked increase in the amplitude of the EPSP (peak amplitude:  $354 \pm 52\%$  of control), but not in the amplitude of the ACh-induced potential.

action potential following PdBu was  $-41.3 \pm 1.7$  mV,  $n=5$  (control,  $40.0 \pm 1.7$ ,  $n=6$ ,  $P > 0.05$ ).

### 3.3. Tests of presynaptic mechanisms following exposure to PdBu

To test the hypothesis that PdBu enhanced synaptic transmission by a presynaptic mechanism, we examined the effects of PdBu on quantal content and the frequency and amplitude of mEPSPs. To measure quantal content, evoked unitary EPSPs were recorded under conditions of low probability of release (low  $\text{Ca}^{2+}$ , high  $\text{Mg}^{2+}$  extracellular solution). A series of stimuli was applied

to the CST under control conditions and after exposure to PdBu. PdBu increased the amplitude of the unitary responses (Figs. 1 and 2) and also decreased the number of synaptic failures. For example, the ratio of the number of evoked spikes to the number of stimuli in one neuron increased after PdBu from a control of 2/280 to 209/321. The quantal content, determined as the mean ratio of EPSP/mEPSP, significantly increased after PdBu to  $2.6 \pm 0.6$  (control  $0.9 \pm 0.2$ ,  $n=5$ ,  $P < 0.05$ ). In many of the neurons, EPSPs could not be recorded because after PdBu, stimulation of the preganglionic fibers in the CST produced action potentials due to the large enhancement of the EPSPs (Fig. 1).

Unitary EPSCs were recorded under voltage clamp using conditions similar to those described for the EPSP. Measurement of the postsynaptic response under voltage clamp reduces both the generation of postsynaptic action potentials commonly found during voltage recordings and an underestimation of the peak EPSP amplitude due to changes in the driving force during postsynaptic depolarizations (Martin, 1955, 1966). In these experiments, quantal content, determined from the mean ratios of the amplitudes, EPSC/mEPSC, was also significantly increased in PdBu from a control value of  $0.5 \pm 0.1$  to  $1.3 \pm 0.2$  ( $n=10$ ,  $P < 0.05$ ). The quantal size, which reflects the amplitude of a single quantum, was not significantly changed following PdBu application (control,  $1.8 \pm 0.2$ ;  $2.2 \pm 0.4$  in PdBu,  $n=10$ ,  $P > 0.05$ ). Histograms of the amplitudes of EPSCs recorded under control conditions and following application of PdBu demonstrated a shift to larger amplitudes following PdBu. The distribution of peaks was fitted with multiples of the unitary synaptic amplitude in the control recordings obtained in the same neuron under conditions of low quantal release (Fig. 4).

Because of the low spontaneous frequency of mEPSPs in the rat SCG (Blackman et al., 1962; Dennis et al., 1971), elevated concentrations of external potassium were used in some experiments to increase the frequency of mEPSPs. In

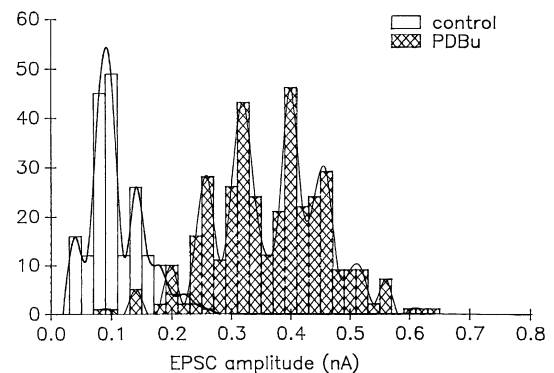


Fig. 4. Mean amplitude histograms of EPSCs recorded in a single neuron in a low-calcium, high-magnesium extracellular solution prior to (open bars) and following (hatched bars) PdBu. PdBu produced a marked increase in the amplitude of EPSCs. Histograms were fitted with a multiple of the unitary mEPSC amplitude recorded in the same neuron.

control solution, the frequency of mEPSPs ranged from 5 to 71 mEPSPs/min. Following PdBu, the frequency increased to a mean value of  $196 \pm 47\%$  of control ( $n=6$ ,  $P<0.05$ ) (Fig. 5). In neurons current-clamped at a membrane potential of  $-100$  mV, the mEPSP amplitude following PdBu was  $4.8 \pm 1.8$  mV ( $n=4$ ), which was not significantly different from control values ( $3.7 \pm 1.1$  mV,  $n=4$ ,  $P>0.05$ ).

The rate of decay of the mEPSP was well fitted with a single exponential function. The time constant of mEPSP decay ( $\tau_{\text{mEPSP}}$ ) was not significantly changed following PdBu application. The  $\tau_{\text{mEPSP}}$  in PdBu was  $44.2 \pm 6.9$  ms ( $n=6$ ) (control  $37.7 \pm 5.4$  ms,  $n=6$ ,  $P>0.05$ ). The time to peak and rate of rise of mEPSPs were also not significantly changed after the addition of PdBu. The time to peak after PdBu was  $7.6 \pm 0.8$  ms (control  $8.2 \pm 1.0$  ms,  $n=6$ ,  $P>0.05$ ) and the rate of rise was  $3.1 \pm 1.0$  mV/ms (control  $2.5 \pm 0.9$  mV/ms,  $n=6$ ,  $P>0.05$ ).

### 3.4. Comparison with tetanic LTP

Previous experiments have demonstrated that tetanic LTP in the rat SCG increases EPSP amplitude without changing the electrical properties of the postsynaptic neuron (Briggs and McAfee, 1988). Tetanic stimulation of the CST for 20 s at 20 Hz increased the amplitude of the EPSP to  $218 \pm 19\%$  ( $n=5$ ) of control. Under two-electrode voltage clamp, the amplitude of the EPSC was increased to  $226 \pm 32\%$  ( $n=10$ ) of the control amplitude (control,  $1.4 \pm 0.1$  nA; tetanic LTP,  $3.2 \pm 0.7$  nA;  $P<0.05$ ). Histograms of EPSCs were generated for each neuron and illustrated a shift to the right of large-amplitude EPSCs following tetanic stimulation (Fig.

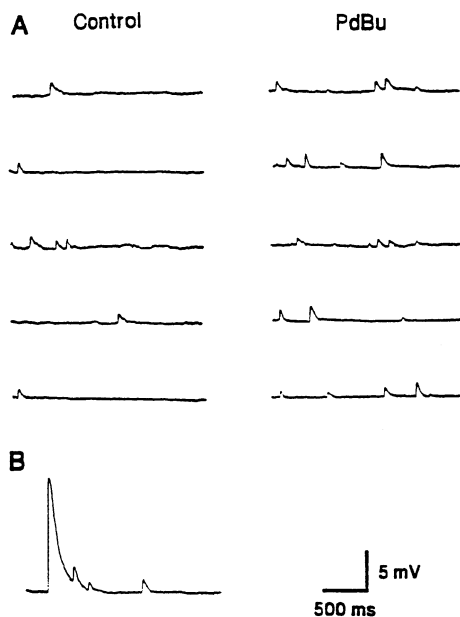


Fig. 5. PdBu increases the frequency of mEPSPs. (A) illustrates mEPSPs recorded in a single neuron before (control) and 20 min after PdBu. (B) shows, in the presence of PdBu, an evoked EPSP followed by several spontaneous mEPSPs.

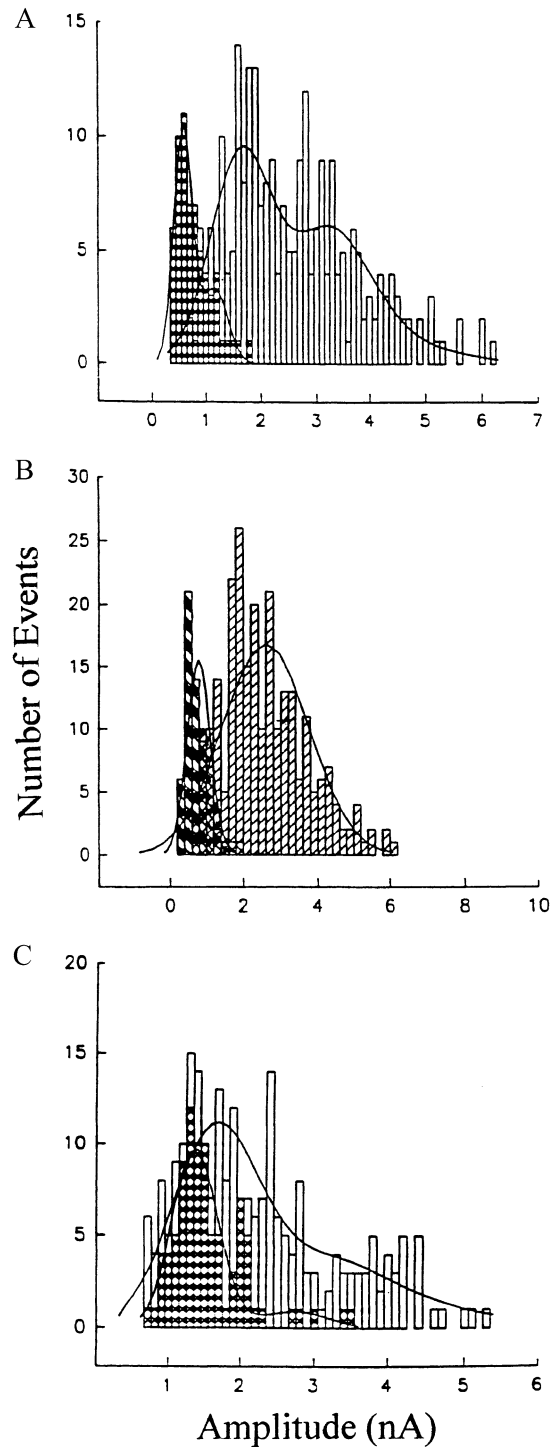


Fig. 6. Amplitude histograms of EPSCs before and after the induction of LTP by tetanic stimulation. The results obtained from the three individual neurons are illustrated in Panels A–C. The histograms are fitted with Gaussian curves and show a significant increase in the mean amplitude of the postsynaptic response after the induction of LTP. The ordinate is the number of events and the abscissa is the amplitude in nanoamperes.

6A–C). There was also a significant increase in the direct quantal content determined from the ratio of mean amplitudes (EPSC/mEPSC) (Fig. 7A and B) (control,  $0.4 \pm 0.1$ ;

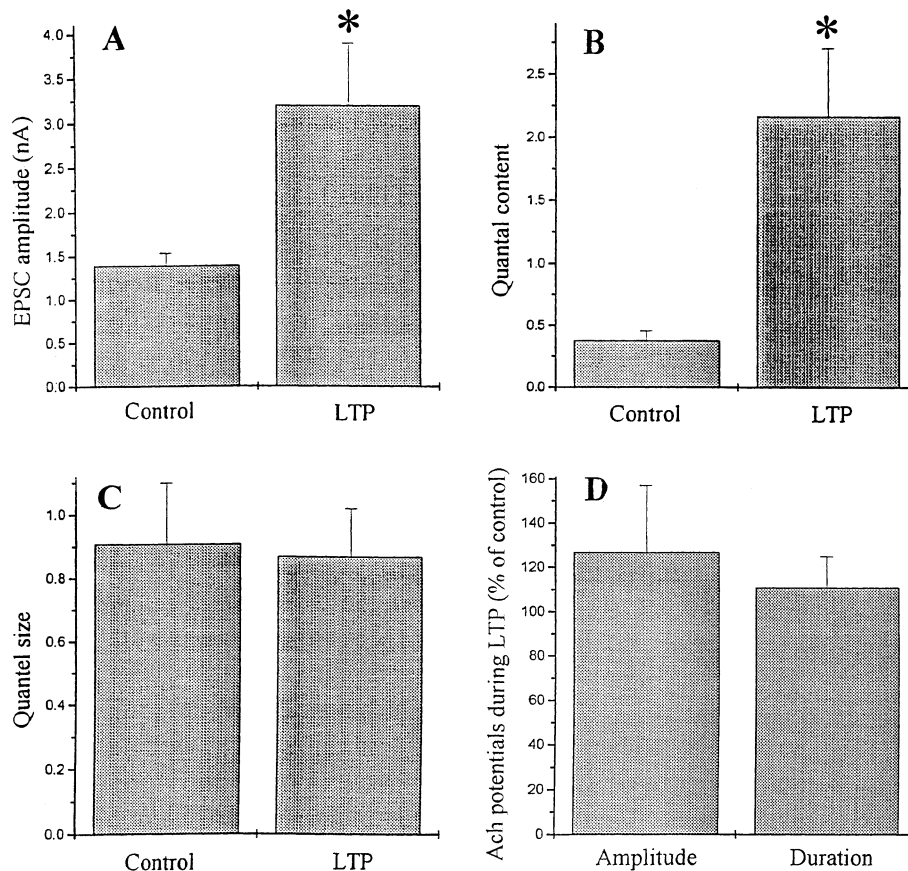


Fig. 7. Tetanically induced LTP increased the mean amplitude of EPSCs (A) as well as the direct quantal content (B). Quantal size was not significantly changed during LTP (C). Sensitivity of the postganglionic membrane to ACh was examined both before and after the induction of synaptic LTP. The induction of LTP did not alter the ACh-induced response amplitude (D). Postsynaptic sensitivity was only examined in neurons with LTP present ( $n = 5$ ). Mean  $\pm$  S.E.M. is presented for the neurons examined both before and after tetanically induced LTP. Asterisks represent significance ( $P < 0.05$ ).

tetanic LTP,  $2.2 \pm 0.5$ ;  $P < 0.05$ ). There was no significant change in either the quantal size (control,  $0.9 \pm 0.1$ ; tetanic LTP,  $0.9 \pm 0.2$ ;  $P > 0.05$ ) or the amplitude of the postsynaptic sensitivity to applied ACh. The amplitude and duration of the ACh potential were  $127 \pm 30.4\%$  and  $111 \pm 13.6\%$  of control ( $P > 0.05$ ), respectively (Fig. 7C and D). There was also no significant change in either the values for the resting membrane potential, input resistance, or the threshold for initiation of an action potential following tetanically induced LTP. The resting membrane potential following tetanic LTP was  $-48.1 \pm 1.6$  mV (control,  $-49.1 \pm 1.3$  mV,  $n = 10$ ,  $P > 0.05$ ). The neuronal input resistance after tetanic LTP was  $83.3 \pm 14.8$  M $\Omega$  (control,  $81.9 \pm 12.9$  M $\Omega$ ,  $n = 7$ ,  $P > 0.05$ ). The threshold for action potential generation was  $-39.8 \pm 1.7$  mV following tetanic LTP (control,  $-40.0 \pm 1.7$  mV,  $n = 6$ ,  $P > 0.05$ ).

#### 4. Discussion

This study examined the properties of the prolonged enhancement of nicotinic synaptic transmission in the rat SCG by the protein kinase C-activating phorbol ester, PdBu,

and by tetanic stimulation of the preganglionic input. Both produced a long-lasting increase in the amplitude of the fast EPSP and EPSC evoked in sympathetic ganglion neurons by preganglionic stimulation.

The potentiation of nicotinic transmission occurred in the absence of changes in the sensitivity of the postganglionic neuron to exogenously applied ACh. However, it is possible that exogenous and endogenous ACh may act on different subsets of postsynaptic nicotinic ACh receptors. If PdBu or tetanic LTP potentiated only *subsynaptic* nicotinic ACh receptors, while exogenous ACh had access only to *extrasynaptic* nicotinic ACh receptors, then the LTP-induced changes may not be detected by exogenous ACh. This seems unlikely, however, because the density of binding sites for [ $^{125}$ I]toxin F (a ligand of neuronal nicotinic ACh receptors), determined by electron microscopic autoradiography in rat sympathetic neurons, is at least 80-fold greater at synaptic than at extrasynaptic sites, suggesting that at any ACh concentration, the synaptic receptors provide the dominant contribution to the response (Loring and Zigmond, 1987; Loring et al., 1988). Moreover, binding of  $\alpha$ -bungarotoxin (a ligand of muscle nicotinic cholinergic receptors) to extrasynaptic nicotinic receptors does not

depress the depolarizing response of the rat SCG to carbachol (Brown and Fumagalli, 1977).

mEPSPs reflect the release of individual quanta from presynaptic terminals in autonomic ganglia (Blackman et al., 1962; Dennis et al., 1971). PdBu and tetanic stimulation increased mEPSP frequency; however, the mean values of mEPSP amplitudes were not significantly different from control. Since there was no correlation between the time to peak and the amplitude of the mEPSPs, we believe it is unlikely that mEPSCs were sampled from electrotonically distant synapses in the neuron. Although recruitment of presynaptic fibers by PdBu cannot be excluded, the similar rates of rise and decay of the unitary EPSPs and EPSCs with respect to control values following both PdBu and tetanic stimulation suggest that recruitment of additional release sites did not affect the analysis under these experimental conditions. Furthermore, neither the threshold nor the latency to the synaptic response was significantly changed following either PdBu or tetanic stimulation.

The mechanism(s) responsible for the production of LTP is clearly not identical among ganglionic synapses. Several second messenger systems and neurotransmitters have been suggested to be involved in the generation of LTP in autonomic ganglia: cAMP (Briggs et al., 1985a; Briggs and McAfee, 1988; Scott and Bennett, 1993), PKC (Bachoo et al., 1992), cyclic guanosine monophosphate, nitric oxide (Alkadhi and Altememi, 1997), calmodulin (Minota et al., 1991), and serotonin (Alkadhi et al., 1996; Southam et al., 1996). For example, in frog, it is clear that neither PKC activation nor cAMP is involved in LTP (Minota et al., 1991). Koyano et al. (1985) demonstrated that LTP in the frog was due to an increase in quantal content in 81% of the ganglion neurons examined and to both presynaptic and postsynaptic changes in 38% of the neurons. Furthermore, the presynaptic changes associated with LTP include an increase in intracellular calcium concentration to increase neurotransmitter release; however, PKC activators potentiated the release, but inhibitors of PKC did not block LTP (Kuba and Kumamoto, 1990; Minota et al., 1991). A form of LTP can be induced by postsynaptic stimulation in the frog (Kumamoto and Kuba, 1983), an effect that cannot be induced in rat SCG neurons (Briggs and McAfee, 1988). cAMP analogs produce LTP in the rat (Briggs et al., 1985a; Briggs and McAfee, 1988; Briggs, 1995). In the cat, LTP involves primarily a postsynaptic change with a minor presynaptic component (Morales et al., 1994).

The effects of both PdBu and tetanic stimulation were to (1) increase the amplitude of the unitary EPSP or EPSCs evoked in ganglion neurons by preganglionic stimulation; (2) markedly increase the proportion of stimuli that evoked EPSPs under conditions of low probability of release; (3) increase the frequency of spontaneous mEPSPs; and (4) increase the direct quantal content. The original work on LTP in the rat SCG suggested that the mechanism was an increase in presynaptic release of ACh based on the findings that the LTP in rat SCG: (1) was present in the absence of

changes in both electrical properties of the postsynaptic neuron and postsynaptic sensitivity; (2) requires presynaptic stimulation; (3) requires extracellular calcium; and (4) is not induced by either antidromic stimulation, direct depolarization of the postsynaptic neuron, or activation of nicotinic receptors (Briggs et al., 1985a; Briggs and McAfee, 1988; Briggs, 1995). They also demonstrated an increase in the release of ACh during LTP without any significant changes in ganglionic content of ACh. (Briggs et al., 1985b; Briggs and McAfee, 1988). Our results are similar to these previous results suggesting a presynaptic site for tetanically induced LTP in SCG and further demonstrate a role for PKC activation in ganglionic LTP.

Several presynaptic mechanisms observed in a variety of synapses have been proposed for the enhancement of synaptic transmission by PKC-activating phorbol esters: (1) modulation of calcium influx through voltage-gated calcium channels (DeRiemer et al., 1985; Strong et al., 1987; Stea et al., 1995; Waters and Smith, 2000); (2) an increase in the sensitivity to calcium of steps involved in stimulus–secretion coupling (Gillis et al., 1996; Yawo, 1999); (3) an increase in vesicle priming for entry of synaptic vesicles into the readily releasable pool (Augustine et al., 1999); (4) an increase in the size of the readily releasable pool of presynaptic vesicles (Parfitt and Madison, 1993; Gillis et al., 1996; Stevens and Sullivan, 1998); (5) a decrease in the rate of clearance of calcium from the cytoplasm; and (6) an increase in calcium influx by broadening the action potential through inhibition of potassium channels (Hoffman and Johnston, 1998). Consistent with a presynaptic action, PKC has been previously shown to phosphorylate a number of presynaptic proteins including cytoskeletal proteins that could influence vesicle trafficking such as VAMP, SNAP25, Munc 18, and the synapsins (Browning and Dudek, 1992; Turner et al., 1999). The precise molecular targets for the generation of LTP are unknown and future studies will be required to differentiate between the potential sites of action of PdBu in preganglionic nerve terminals.

Although potentiation of ganglionic transmission in the rat SCG occurs regularly using extracellular recording techniques, such as the CAP, when examined with intracellular recording at the single cell level, LTP is present in only approximately 70% of neurons (Briggs and McAfee, 1988; Koyano et al., 1985). Since not all individual neurons produce LTP under a given set of experimental conditions, there may be several mechanisms for the generation of LTP that can only be elucidated at the single cell level. We have previously demonstrated that both tetanic stimulation-induced and PdBu-induced potentiation of the CAP of the rat SCG are blocked by inhibitors of protein kinase C. Furthermore, not only do phorbol esters, which do not activate PKC, not produce LTP but either previous exposure to PdBu or tetanic stimulation mutually precludes the subsequent generation of LTP by the other mechanism (Bachoo et al., 1992). These results suggest that PKC activation is common to tetanically and PdBu-induced

LTP and may activate the same intracellular pool of PKC. The similarities in the single cell electrophysiological analysis of enhancement of synaptic transmission produced by PdBu and tetanic stimulation in the present study further suggest that activation of PKC is a contributor to the presynaptic mechanism in rat SCG.

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