Colchicine affects protein kinase C-induced modulation of synaptic transmission in cultured hippocampal pyramidal cells

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Abstract Spontaneous excitatory postsynaptic currents (sEPSC) through AMPA-type channels were recorded on cultured hippocampal pyramidal neurons by means of the whole-cell patch-clamp technique. The protein kinase C (PKC) agonist 4β-phorbol 12-myristate 13-acetate (4β-PMA) produced a long-lasting increase in sEPSC frequency not mimicked by the inactive analogue 4α-PM and blocked by the protein kinase inhibitor staurosporine. The 4β-PMA-induced change in sEPSC frequency occurred without detectable change in $[Ca^{2+}]_i$. After treatment with the microtubule-disrupting agent colchicine, 4β-PMA caused a small and transient increase in sEPSC frequency. It is concluded that colchicine affects the PKC-induced functional plasticity of nerve cells most likely by disturbing the axonal transport.

Key words: Hippocampus; Protein kinase C; Axonal transport; Colchicine; Microtubule; Exocytose

1. Introduction

Amino acid neurotransmitters are packaged into small vesicles that can release their content into the synaptic cleft even in the absence of depolarization of the presynaptic membrane. This leads to activation of postsynaptic receptors generating spontaneous excitatory (sEPSC) or inhibitory (sIPSC) postsynaptic currents. The mechanisms underlying this spontaneous quantal release are not elucidated. However, the sEPSC (or sIPSC) frequency can be modulated by different protein kinase (PK) activators. For instance, forskolin and phorbol esters, known as cAMP-dependent PKA and PKC activators, respectively, produce a long-lasting modulation of spontaneous transmitter release [1–5].

Microtubules are found in most cell types, including neurons, and are thought to be involved in many physiological functions like the intracellular transport of vesicles and mitochondria [6,7]. The axonal transport can be blocked by the microtubule-depolymerizing agent colchicine [6,8]. The action of this plant alkaloid could impair the functional properties of nerve cells since, for instance, it inhibits the transport of amine storage vesicles in sympathetic nerves [9]. Thus, uncoupling the nerve terminals from its axon may effect the responsiveness of a neuronal cell to agents acting at a presynaptic site and modulating synaptic transmission. This hypothesis was tested on cultured rat hippocampal cells. The phorbol ester 4β-PMA was used to produce a long-lasting increase in sEPSC frequency. This effect that occurred without detectable change in $[Ca^{2+}]_i$ was strongly attenuated after colchicine treatment. The inactive analogue β-lumicolchicine failed to mimick this effect showing that colchicine impairs the PKC-induced synaptic plasticity of cultured hippocampal cells, most probably by blocking the axonal transport.

2. Materials and methods

2.1. Cell cultures

Hippocampal cell cultures were prepared from 3- to 5-day-old Sprague-Dawley rats as described in detail elsewhere [10–12]. Briefly, the cell dissociation procedure consisted of a trypsin treatment (3.4 mg/ml trypsin type XI+0.9 mg/ml DNase type IV) followed by a gentle mechanical trituration in a Ca^{2+}-free Hanks' solution supplemented with 12 mM $MgSO_4$, 0.4 mg/ml DNase and 3 mg/ml bovine serum albumin. After centrifugation (2 times 80×g for 15 min) the dissociated cells were plated on poly-L-ornithine-coated coverslips in a small area confined by a cloning cylinder. Hippocampal cells were kept in a small volume (≈160 μl) of minimal essential medium supplemented with 29.2 mg/ml glutamax, 6000 mg/l glucose, 25 mg/l insulin, 100 mg/l transferrin, 5 mg/l gentamycin, and 10% fetal calf serum (FCS). Cells were maintained in a incubator at 37°C (6.5% CO_2). To reduce astrocyte growth, cytosine β-D-arabinofuranoside (3 μM) was added at day 2. At that time 2% of B-27 supplement was then added to the culture medium and the FCS was reduced to 5%. Some culture dishes were treated with colchicine (10 μM) or β-lumicolchicine (10 μM) for 9–25 h prior to the electrophysiological experiments.

2.2. Electrophysiology

sEPSCs were recorded on hippocampal pyramidal cell bodies by means of the whole-cell configuration of the patch-clamp technique [13] with an EPC7 amplifier (List Electronics, Germany). Thick-wall glass pipettes (1.5–3.5 MΩ) were filled with a solution containing (in mM): Cs-gluconate or CsF, 135; $MgCl_2$, 2; EGTA, 10; HEPES, 10; Na_2ATP, 2–5; GTP, 0.2 (pH 7.2). Throughout this study the cells were voltage-clamped at −50 mV and the sEPSCs were filtered at 1–2 kHz and sampled on-line at 10 kHz on the hard disk of a microcomputer driven by the CED software (version 6.0, Cambridge Electronics, UK) [14]. Cells were kept on the stage of an inverted microscope and bathed with a medium consisting of (in mM): $NaCl_2$, 135; KCl, 5; $MgCl_2$, 2; $CaCl_2$, 2 or 10; glucose, 30; HEPES, 10 (pH 7.4). Drug-containing solutions were added by a gravity-driven system perfusing the entire chamber. All the experiments were performed at room temperature.

sEPSCs have been analyzed as described before [14] with a fully automated computer program written by Dr. A. Dityatev (University of Hamburg, Germany), based on the algorithm of Ankri et al. [15].

2.3. $[Ca^{2+}]_i$ measurements

Changes in the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) were measured with the fluorescent probe Fluo-3. Hippocampal cells were incubated with 5–10 μM Fluo-3/AM for 30–50 min. The experiment procedures, carried out with a confocal Laser Scan Microscope (LSM410, Zeiss AG, Germany), have been explained in detail elsewhere [12,14].

2.4. Materials

Trypsin (type XI), cytosine β-D-arabinofuranoside, the DNase (type IV), and bicuculline were purchased from Sigma Chemie AG (Buchs, Switzerland). 4β-PMA was from Sigma (St. Louis, MO). The B-27 supplement was from Gibco BRL (Life Technologies, Basel, Switzer-

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land), the minimal essential medium was from Gibco BRL or Sigma Chemie AG (Buchs, Switzerland). TTX was from Calbiochem Biochemicals (Lucern, Switzerland). CNQX was purchased from Tocris Cookson (St. Louis, MO), 4α-PMA from ICN Biomedicals (Meckenheim, Germany), and staurosporine from Fluka AG (Buchs, Switzerland). Fluo-3 was from Molecular Probe (Leiden, The Netherlands), colchicine and β-lumicolchicine from Serva (Wallisellen, Switzerland).

3. Results

Throughout this study, measurements were done 9–15 days after the plating of the hippocampal cells. sEPSCs were recorded from an holding potential of −50 mV in the presence of 0.1–0.2 μM TTX or 100 μM $CdCl_2$ to block propagated action potentials and evoked transmitter release. The sampling of the sEPSCs started 2–3 min after the establishment of the whole-cell configuration. Postsynaptic currents were sampled for 3.1–6.2 s every minute for 5–7 min before the addition of 4α- or 4β-PMA.

Fig. 1A shows representative current traces of sEPSCs recorded in the presence of 100 μM Cd^{2+}. The addition of the AMPA channel antagonist 6-cyano-nitroquinoxaline-2,3-dione (CNQX, 10 μM) reversibly blocked the sEPSCs. Under the experimental conditions (2 mM $MgCl_2$ in the external and pipette solutions), the NMDA channels were completely inhibited. The addition of 100 nM 4β-PMA increased the sEPSC frequency (Fig. 1B). This effect could also be completely and reversibly inhibited by CNQX. Thus, the basal activity and the 4β-PMA-induced increase of sEPSC frequency are both mediated by CNQX-sensitive glutamate receptors. Fig. 1C shows the time-dependence of the 4β-PMA action. The sEPSC frequency increased rapidly after the addition of the PKC activator (Fig. 1C, ■) and could remain elevated for up to 90 min. This long-lasting change in sEPSC frequency was never observed with 4α-PMA (Fig. 1C, □, and Fig. 1E). Ten minutes after its addition, 4β-PMA produced a 4–5-fold increase in sEPSC frequency (Fig. 1E). This effect was blocked by the protein kinase inhibitor staurosporine. Similar effects of 4β-PMA were observed when Na^+ channels were inhibited with TTX (Fig. 1E).

4β-PMA did not affect the kinetic properties of sEPSCs. Mean rise times (10–90%) were 0.88 ± 0.02 ms ($n = 111$) and 0.90 ± 0.02 ms ($n = 442$) (means ± SEM) in control and 4β-PMA-treated cells. Amplitude distribution histograms presented in Fig. 1D illustrate the change in sEPSC frequency. They were constructed after sampling sEPSCs for 24.8 s in control and in 4β-PMA-containing solutions, respectively. sEPSC amplitudes were 12.3 ± 0.5 pA ($n = 111$) and 14.3 ± 0.4 pA ($n = 442$) in control and 4β-PMA-treated cells. The inset shows amplitude distribution histograms from the same experiment but constructed with an equal number ($n = 111$) of sEPSCs for each experimental condition. Both histograms are perfectly superimposable, indicating that 4β-PMA had no effect on the amplitude of sEPSCs.

Taken together, these data show that 4β-PMA up-regulated the spontaneous release of glutamatergic synaptic vesicles in hippocampal pyramidal cells most probably by activating PKC. Furthermore, the increase in spontaneous transmitter release occurs even after the blockage of voltage-gated Ca^{2+} channels (i.e. in Cd^{2+} solution) or after preventing cell depolarization (i.e. in TTX solution). This effet was without detectable change in the kinetic properties or amplitudes of the sEPSCs, indicating a presynaptic site of action of 4β-PMA [3,4,16].

Since the evoked release of neurotransmitters critically depends on an influx of Ca^{2+} through voltage-gated Ca^{2+} channels [17,18], the role of the external and internal $[Ca^{2+}]$ on the 4β-PMA-induced modulation of synaptic transmission has also been investigated. Experiments similar to the ones reported in Fig. 1 were also carried out in the presence of 10 mM external $[Ca^{2+}]$ (Fig. 2A) instead of 2 mM $[Ca^{2+}]$ (Fig. 1). In both cases 4β-PMA produced a ≈4-fold increase in sEPSC frequency. If hippocampal cells were incubated with the cell permeant Ca^{2+}-chelator BAPTA/AM (2 μM at 37°C for >20 min) the sEPSC frequency was increased to a similar extent by 4β-PMA (Fig. 2A). This shows that 4β-PMA-induced modulation of synaptic transmission was not affected by changes in $[Ca^{2+}]_o$ [4] and the PKC activator does not exert its action by an increase in basal $[Ca^{2+}]_i$. This was further tested by measuring $[Ca^{2+}]_i$ with the fluorescent probe Fluo-3. The measurements were done on cell bodies and on dendritic networks. A field stimulation (20 Hz, 1 ms pulse duration) increased $[Ca^{2+}]_i$ in cell bodies (Fig. 2B) and dendrites (Fig. 2C, solid lines). The $[Ca^{2+}]_i$ returned to basal levels after cessation of the stimulus. This stimulus-induced $[Ca^{2+}]_i$ rise was completely and reversibly inhibited in the presence of TTX, Cd^{2+}, and CNQX (Fig. 2B,C, dotted lines). 4β-PMA was then perfused together with these blockers. Under these conditions the basal level of $[Ca^{2+}]_i$ remained stable indicating that 4β-PMA does not increase $[Ca^{2+}]_i$ (Fig. 2D,E).

The plant alkaloid colchicine blocks axonal transport of vesicles [6,7,19]. Experiments were performed to assess the role of the microtubule-associated transport on the long-lasting 4β-PMA-induced change in sEPSC frequency.

Hippocampal cells were incubated with colchicine (10 μM) or its inactive analogue β-lumicolchicine (10 μM) for 9–25 h. Colchicine-treated cells had the same morphology as β-lumicolchicine-treated cells. Cell capacitance values were 36.6 ± 4.6 pF ($n = 9$) and 32.9 ± 3.2 pF ($n = 3$) in colchicine- and β-lumicolchicine-treated cells, respectively, indicating no change in cell size. Colchicine did not affect the basic exocytotic process because sEPSCs (Fig. 3A) could be observed even after a 24 h

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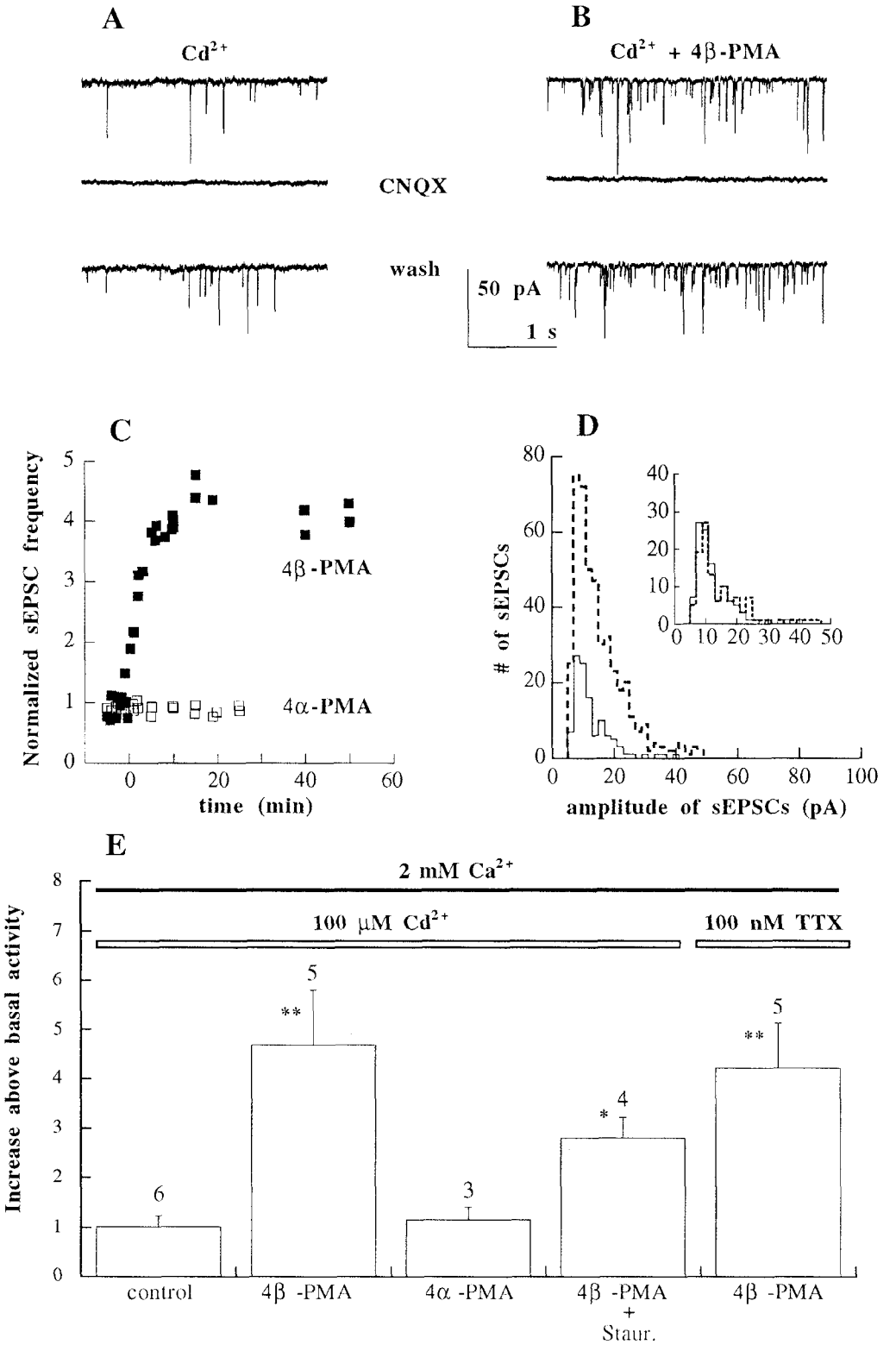
Fig. 1. 4β-PMA increases the sEPSC frequency. Cultured hippocampal pyramidal cell bodies were voltage-clamped at −50 mV. The external medium contained 2 mM Ca^{2+} and 100 μM Cd^{2+}. A: sEPSCs were reversibly blocked by CNQX (10 μM). B: 4β-PMA (100 nM) increased the sEPSC frequency. This effect was reversibly inhibited by CNQX (10 μM). C: Time-dependence of 4β-PMA action. The graph shows normalized sEPSC frequencies before and during the addition of 4α- (□) or 4β-PMA (■). The drugs were added at time 0. D: Amplitude distribution histograms of sEPSCS sampled for 24.8 s before (solid line, $n = 111$ sEPSCs) and during 4β-PMA application (dotted line, $n = 442$ sEPSCs). The inset shows similar amplitude distribution histograms from the same experiment but with an equal number of sEPSCs ($n = 111$) for each group (control: solid line; 4β-PMA: dotted line). The data reported in (A,B), (C) and (D) were collected from 3 different cells. E: The graph shows the change in sEPSC frequency, in Cd^{2+}-containing solution, measured 10 min after the addition of the vehicle (0.01% DMSO, control), 4β-PMA (100 nM), or 4α-PMA (100 nM). Some cells were pretreated with staurosporine (500–800 nM for 15–35 min, staur.) or TTX (100 nM) before the addition of 4β-PMA. The number of cells tested is indicated above each bar (means ± SEM). $^*P < 0.05$, $^{**}P < 0.01$ vs. control.

treatment of cells with the plant alkaloid. Since colchicine treatment has been reported to affect ligand-gated ion channel properties expressed in *Xenopus* oocytes [20], the kinetic characteristics of the sEPSCs were analyzed. The rise-times were 1.1 ± 0.02 ms ($n = 161$) and 1.2 ± 0.04 ms ($n = 161$) in colchicine- and β-lumicolchicine-treated cells, respectively. Amplitude distribution histograms had comparable peaks (Fig. 3A), indicating that the treatment did not affect postsynaptic receptor functions (19.2 ± 1.2 pA ($n = 161$) in colchicine- vs. 17.7 ± 1.4 pA ($n = 161$) in β-lumicolchicine-treated cells).

When cultured cells were incubated for 16–25 h with 10 μM β-lumicolchicine and washed with a normal saline the external application of 100 nM 4β-PMA increased sEPSC frequency 5.6 ± 1.5 fold ($n = 3$) in a time-dependent manner as illustrated



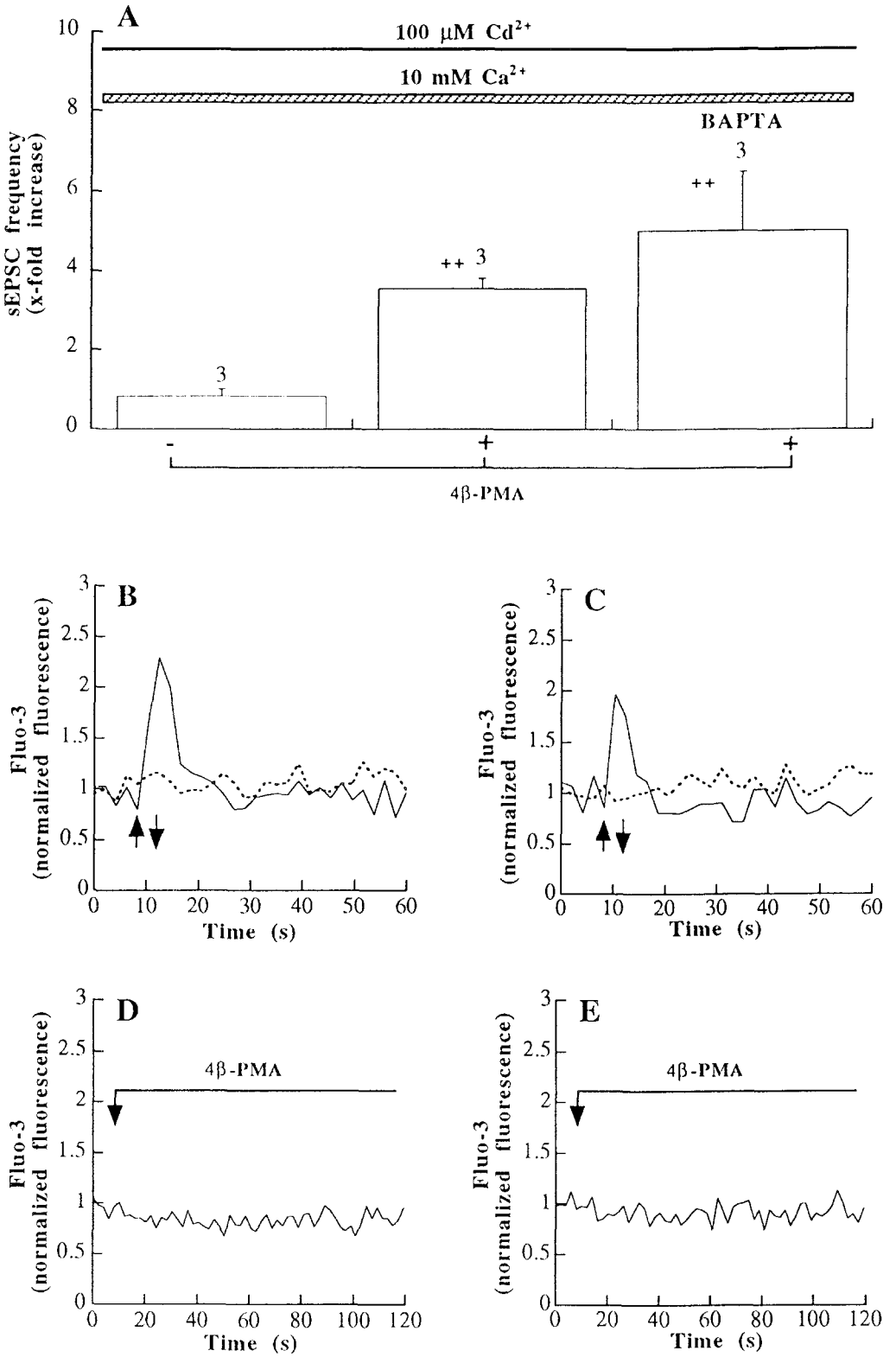


Fig. 2. Dependence of 4β-PMA action on $[Ca^{2+}]$. A: The graph shows the *x*-fold increase in sEPSC frequency measured 10 min after the addition of 4β-PMA (+) or its vehicle (−). Cells were superfused with 10 mM $[Ca^{2+}]_o$. Cd^{2+} (100 μM) was used to block voltage-gated Ca^{2+} channels. Some cells were incubated at 37°C in the presence of the cell permeant Ca^{2+}-chelator BAPTA/AM (2 μM). The number of cells tested is indicated above each bar (means ± SEM). B–D: $[Ca^{2+}]_i$ signals were recorded by the means of the fluorescent dye Fluo-3. The cells were incubated with 5–10 μM of the membrane permeable Fluo-3/AM for 30–50 min. $[Ca^{2+}]_i$ changes were measured in cell bodies (B,D) and in the dendritic network (C,E). Hippocampal cells were field stimulated at 20 Hz for 4–5 s (arrows) without (solid lines, B,C) or with 0.2 μM TTX +100 μM Cd^{2+} +10 μM CNQX (dotted lines, B,C). D,E: When indicated (downward arrow) 4β-PMA (100 nM) was added and continuously superfused in the presence of the blockers (TTX, Cd^{2+}, CNQX). D: Cell body. E: Dendrites. Similar results were obtained on 4 cells from 3 cell cultures. $^{++}P < 0.01$ vs. control (no added 4β-PMA).

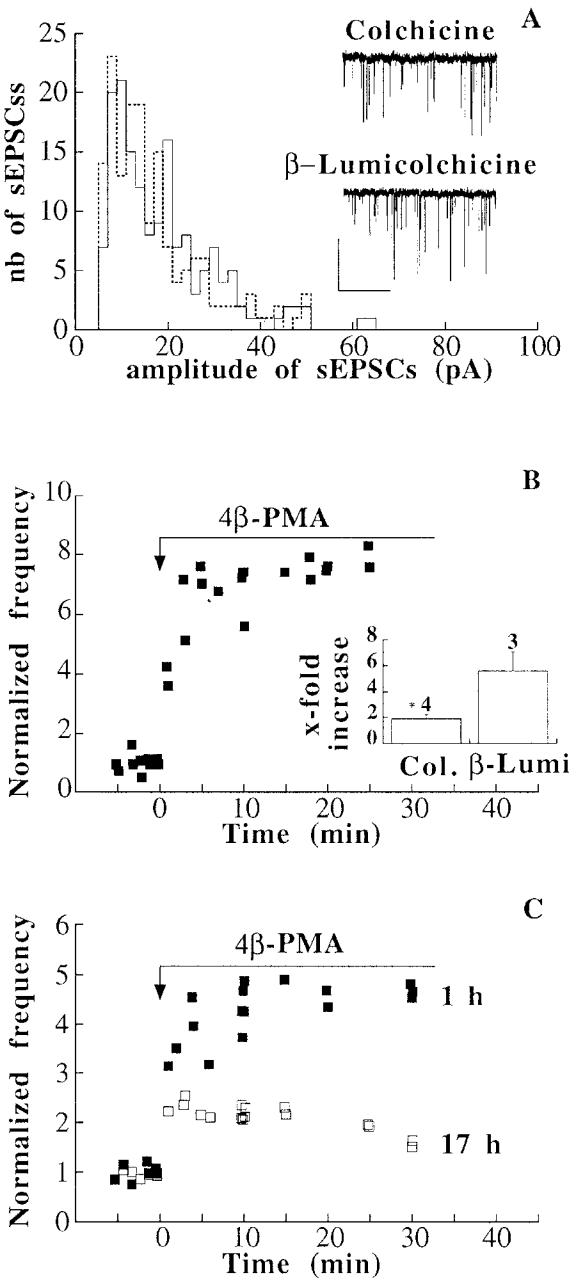


Fig. 3. The long-lasting increase in sEPSC frequency induced by 4β-PMA is inhibited by colchicine. A: Amplitude distribution histograms of sEPSCs recorded on cells pretreated with colchicine (10 μM, 14 h, solid line) or with its inactive analogue β-lumicolchicine (10 μM, 17 h, dotted line). In the inset, sEPSCs measured after the indicated treatment are shown. Scale bars: 10 pA, 1 s. B: Time-dependence of 4β-PMA action. β-Lumicolchicine-treated cells responded to 4β-PMA like non-treated cells (see Fig. 1). 4β-PMA was added at time 0. The plot in inset shows the *x*-fold increase in sEPSC frequency induced by 100 nM 4β-PMA on colchicine (Col.)- and β-lumicolchicine (β-Lumi.)-treated cells. The number of cells tested is indicated above each bar. The external medium contained 100 μM Cd^{2+} and 100 nM 4β-PMA. The cells were pretreated for 9–24 h (Col.) and 16–25 h (β-Lumi) (means ± SEM). $^{*}P < 0.05$ vs. β-lumicolchicine. C: Cultured hippocampal cells were incubated with 10 μM colchicine for 1 (■) or 17 h (□). In the latter case, 4β-PMA produced only a small and transient increase in sEPSC frequency.

in Fig. 3B. In the presence of colchicine, this effect was not only considerably reduced after a prolonged treatment of the cells (9–24 h, $n = 4$) but within 20–40 min the sEPSC frequency returned to the level before 4β-PMA application. Thus, 4β-PMA produced only a transient 2-fold change in sEPSC frequency (1.9 ± 0.3 ($n = 3$); Fig. 3C, □) in colchicine-treated cells. Short-time exposures (1–3 h) to colchicine failed to mimick this inhibitory effet (Fig. 3C, ■).

4. Discussion

The phorbol ester 4β-PMA produced a long-lasting increase in sEPSC frequency, most likely through activation of PKC [1,3–5]. A previous study showed that this effect requires an influx of Ca^{2+} through voltage-gated Ca^{2+} channels [16]. However, the experiments reported with the non-specific voltage-gated Ca^{2+} channel blocker Cd^{2+} (Fig. 1) do not support this notion. A similar conclusion was reached for the effect of the phorbol ester PBDu on sIPSCs [5]. The change in sEPSC frequency occurs in absence of $[Ca^{2+}]_o$ [4], in low and high $[Ca^{2+}]_o$, and in BAPTA/AM-loaded cells. Furthermore, 4β-PMA exerts its action without any detectable change in $[Ca^{2+}]_i$. These results show that phorbol esters can modulate synaptic transmission in a Ca^{2+}-independent manner. In chromaffin cells 4β-PMA increases the size of the pool of readily releasable vesicles [21]. This could explain the 4β-PMA effects in the present study.

The most striking result is the reduction of the 4β-PMA-induced enhancement of sEPSC frequency after exposure to colchicine (Fig. 3). Short-term exposures to colchicine failed to produce this effect. Furthermore, β-lumicolchicine-treated cells responded to 4β-PMA like non-treated cells, indicating the specificity of the colchicine action. The data presented in Fig. 3 are not due to postsynaptic alterations for the following reasons: (1) kinetics and amplitude distribution histograms of sEPSCs were similar in β-lumicolchicine- and colchicine-treated cells; and (2) microtubules are sometimes found in the neck of dendritic spines but are absent from the bulb that is enriched in actin filaments [6,22]. Thus, the ability of colchicine to prevent the 4β-PMA-induced enhancement of sEPSC frequency is most likely a presynaptic effect. One possible explanation is a colchicine-induced disruption of the microtubule-network [19] and, thereby, a blockade of the axonal transport of vesicles [6,7]. The occurrence of sEPSCs indicates that the treatment did not block the exocytotic machinery. This is in agreement with recent work showing that colchicine does not affect the cycling of presynaptic vesicles stained with the styryl dye FM1-43 [23]. Colchicine-treated cells underwent normal activity-dependent exo/endocytotic cycles, indicating that colchicine treatment did not block the vesicle mobilization at the release sites [23]. Thus, the effects reported in the present study may be the consequence of colchicine-induced changes that take place upstream of the nerve terminal. Presynaptic proteins, synthetized in the cell body, migrate down the axon [24]. The colchicine-induced block of the axonal transport may have interrupted this flow, altering the nerve terminal content of newly synthetized proteins. Numerous cytoskeleton, cytosolic and presynaptic proteins can be phosphorylated by PKC. For instance, several endogenous PKC substrates are present in hippocampal synaptosomal preparations [25]. Then, the reduction of the 4β-PMA-induced in-

crease in sEPSC frequency could be a consequence of a decreased level of PKCs and/or PKC substrates.

The site of synaptic vesicle formation is not known. They can originate from the nerve terminal or the cell body, e.g. via the trans-Golgi network [24]. In the light of previous experiments [21], it is tempting to speculate that the modest increase in sEPSC frequency produced by 4β-PMA in colchicine-treated cells is due to a reduced number of presynaptic vesicles.

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