Hiroyasu Satoh

—Original—

Effects of Protein Kinase C on the L-Type Ca^{2+} Channel in Single Rabbit Sino-Atrial Nodal Cells: Analysis by Patch-Clamp Technique

Hiroyasu Satoh

Department of Pharmacology, Nara Medical University, Kashihara, Nara 634, Japan

Key words: Protein Kinase C, H-7, Whole-cell clamp, Cell-attached patch clamp, Single sino-atrial nodal cell

Abstract

Effects of phorbol esters (stimulator of protein kinase C) on the L-type Ca^{2+} current in isolated single rabbit sino-atrial (SA) nodal cells were examined. In whole-cell voltage-clamp experiments, 12-0-tetradecanoylphorbol-13-acetate (TPA) at 10^{-7} M inhibited the L-type Ca^{2+} current. Pretreatment with H-7 (10^{-5} M), an antagonist of protein kinase C (PK-C), did not modify the TPA-induced depression. The time course of inactivation process for the L-type Ca^{2} current was slowed. In cell-attached patch-clamp experiments, TPA (10^{-7} M) profoundly decreased the probability of the opening of unitary L-type Ca^{2+} channels (a dihydropyridine-sensitive). The conductance was unaffected (18±1 pS, n=5). Even H-7 (10^{-5} M) alone tended to inhibit the opening of the channels. Addition of TPA (10^{-7} M) to the H-7-containing solution caused further decrease in the opening. On the other hand, 4-alpha-phorbol-12, 13-didecanoate (not a PK-C activator) had no effect on the Ca^{2+} channels. These results indicate that the PK-C stimulation decreases the opening of L-type Ca^{2+} channels of the SA nodal cell membranes.

Introduction

It is known that protein kinase C (PK-C) is an important component of cellular signal transductions for biological activators of substances[1]. PK-C is Ca^{2+}- and phospholipid-dependent protein kinase, and recently PK-C independent of Ca^{2+} has also identified. A stimulant specifically binds to a receptor of cell surface, which activates a phospholipase C for phosphatidylinositol (PI) located on the cytosolic face of the plasma membrane. The active enzyme releases phosphatidyl-inositol-4, 5-biphosphate (PIP_2) into the cytosol, and leaves myo-inositol-1, 4, 5-triphosphate (IP_3) and diacylglycerol (DG) in the membrane. IP_3 mobilizes Ca^{2+} stored in the sarcoplasmic reticulum (SR)[2),3)]. DG activates PK-C by stabilizing its insertion into the membrane. Activated PK-C then phosphorylates substrate

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proteins, and thereby the physiological responses are elicited[2),4)].

It has already been reported for so many physiological effects of PK-C stimulation; (1) increase in RNA, protein and DNA synthesis[5),6)], (2) platelet aggregation and phosphorylation of a peptide in blood platelets[7),8)], (3) increases in Na^+-K^+ pump activity and Na^+-H^+ exchange[9),10)]. In our studies, the PK-C stimulation caused a negative inotropic effect in guinea-pig ventricular muscle[11)], and caused a negative chronotropic effect and elevated cellular Ca^{2+} concentration in rabbit sino-atrial (SA) node cells[12-14)]. Furthermore, stimulation of PK-C increased the L-type Ca^{2+} current ($I_{Ca(L)}$) in rabbit SA node cells[12)], whereas the stimulation decreased it in guinea-pig ventricular and vascular smooth muscle cells[14-16)]. Thus, the effects of PK-C stimulation on the $I_{Ca(L)}$ current was controversial.

In the present experiments, I wanted to examine how TPA modulates the L-type Ca^{2+} channels in rabbit SA nodal cell membrane using a patch-clamp technique. To confirm the actions induced by PK-C activation, both PDD (which lacks an activator of PK-C) and H-7 (an inhibitor of PK-C) were used.

Materials and Methods

Cell preparation

Rabbits either sex, weighing 1.5-2.0 kg, were anaesthetized with intraveneous injection of sodium pentobarbital (30 mg/kg). After the rabbit was exsanguinated, the chest was opened. The heart was dissected out and immersed in normal Tyrode's solution, according to previous reports[13),14)]. The SA node strips were cut further in pieces in the nominally Ca^{2+}-free Tyrode's solution. The spontaneously beating heart was ceased. Then, the perfusate was switched to low-Ca^{2+} (30-60 μM) Tyrode solution containing 0.1 mg/ml collagenase (Type I, Sigma Chemical Co., St. Louis, MO) for about 30-35 min. The preparations were transferred to high-K^+ and low-Cl^- solution (KB solution), and stored at 4℃ for 1 h.

Whole-cell voltage-clamp recording

Whole-cell patch clamp experiments[14-18)] were done using glass patch pipettes. The tip diameters was 3-5 μm and the resistance was 3-10 MΩ. The membrane capacitance was 25.8 ± 4.0 pF (n = 14). Current and voltage records were stored on magnetic tape for computer analysis (NEC 98 series). The compositions of the modified Tyrode's solution was as follows (mM); NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2P0_4 0.33, glucose 5.0, HEPES 5.0 (pH 7.4). The pipette (intracellular) solution contained (mM); CsOH 110, aspartic acid 90, CsCl 20, K_2ATP 5, creatine phosphate 5, EGTA 5, $MgCl_2$ 1, HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, Wako Pure Chemical Industries, Ltd., Osaka, Japan) 5, and cyclic AMP 0.05. Intracellular pH was adjusted to 7.2 and pCa was 7.2. The values represent mean±S. E. M.

Unitary current recording

To record single channels, cell-attached patch-clamp experiments were performed[14),17)]. The resistance of the patch electrode was 3-5 MΩ, and the tip of the electrode was coated with Sylgard (KE106, Shin-etsu Chemical Co.). Unitary current traces were stored on a video recorder (BR6400, Victor) using a PCM converter system (RP-880, NF Electronic Circuit Design, Tokyo). The data were analysed on an Hitachi E600 computer. Current traces were filtered with a cut-off fre-

quency of 1 kHz for plotting (FV-625, NF). The membrane potential of the patch was calculated as the difference between the resting potential and the pipette potential. The pipette solution contained (mM): $BaCl_2$ 50, cholin-Cl 70, glucose 5, HEPES 0.5, Bay K 86442 10^{-6} M, and tetrodotoxin 10^{-5} M (pH 7.4). All experiments were performed at 37℃.

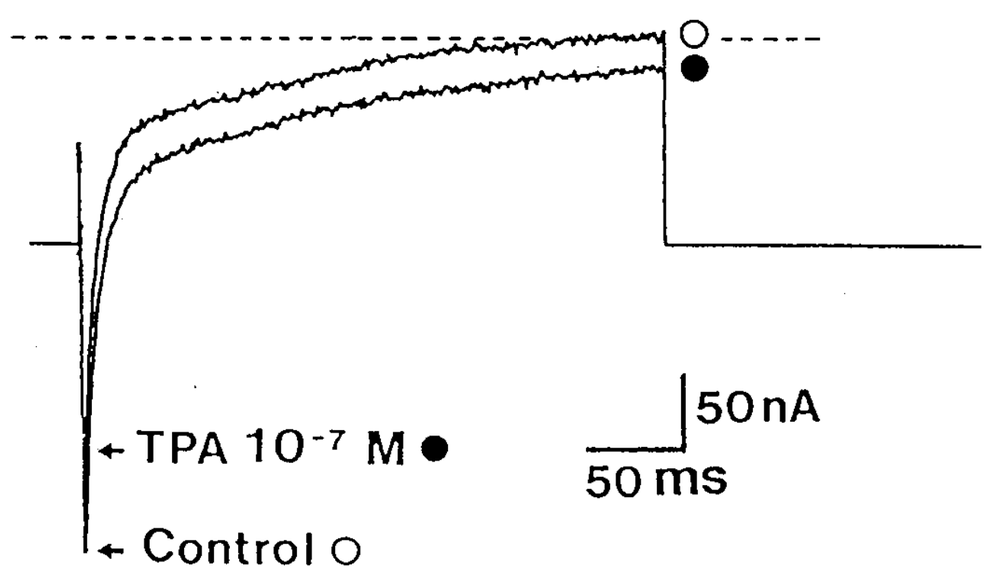

Fig. 1. Depression in the L-type Ca^{2+} current by TPA in single sino-atrial nodal cell. Whole-cell voltage clamp experiment was exerted. Test pulse (for 300 ms) was applied to 0 mV from a holding potential of −40 mV. Phorbol ester, 12-0-tetradecanoyl-phorbol-13-acetate (TPA) was added to the bath solution. Symbols used are control (open circles) and TPA 10^{-7} M (filled circles). K^+ in the pipette solution was replaced by Cs^+. Dashed line represents zero current level.

Drugs

Drugs used in this study were 12-0-tetradecanoylphorbol-13-acetate (TPA) and 4-alpha-phorbol-12, 13-didecanoate (PDD) (Sigma Chemical Co., St. Louis, MO, U. S. A). Both phorbol esters were dissolved in dimethyl sulfoxide (DMSO), which was diluted 7,000–20,000 times in the perfusion medium, and stocked at −10℃. Also, H-7[1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine dihydrochloride] (Seikagaku Kogyo Co., Tokyo) and tetrodotoxin (TTX, Sankyo Co., Tokyo) were dissolved in distil water. Since solution in the bath were exchanged within 1 min and the effects of drugs completely reached a steady state within 3 min, the data were obtained for 3–5 min after exchanging to the new solution.

Results

L-type Ca^{2+} current

Effects of TPA on the L-type Ca^{2+} current ($I_{Ca(L)}$) in isolated SA nodal myocytes were examined by whole-cell patch-clamp technique (Fig. 1). Test pulse was applied to 0 mV for 300 ms duration from a holding potential of −40 mV. The peak of $I_{Ca(L)}$ current was −325±11 pA (n=7), and was sensitive to nifedipine (10^{-6} M). TPA (10^{-7} M), a PK-C activator, was added to the bath solution. TPA depressed the $I_{Ca(L)}$ current. The percentage inhibition were 20.3±1.5% (n=7, P<0.01). The time course of inactivation process for $I_{Ca(L)}$ was slowed in the presence of TPA (10^{-7}M). Additional application of H-7 (10^{-5} M) (an inhibitor of PK-C) had little or no effect on the depressant $I_{Ca(L)}$ in all of 6 cells (but be not shown).

Single L-type Ca^{2+} channels

To examine the effects of PK-C activation induced by TPA on unitary L-type Ca^{2+} current, cell-attached patch clamp experiments were performed. Depolarizing test pulses of 70 mV for 300 ms were applied from the resting potential (−80 mV). The conductance was unaffected (18±1 pS, n=5). As shown in Fig. 2A and B, TPA (10^{-7} M) reduced the opening probability by 21.3±3.3% (n=6, P<0.01). In another cell, H-7 (10^{-5} M) alone tended to decrease the opening probability (by 6.2±1.6%, n=5, P>0.05) (Fig. 3A and B). Addition of TPA (10^{-7} M) in the presense of H-7 (10^{-5} M) potentiate to decrease the opening by 43.2±2.4% (n=5, P<0.01) (Fig. 3C). No antagonistic action by H-7 was observed in all of six cells.

On the other hand, PDD (10^{-7} M) (a phorbol

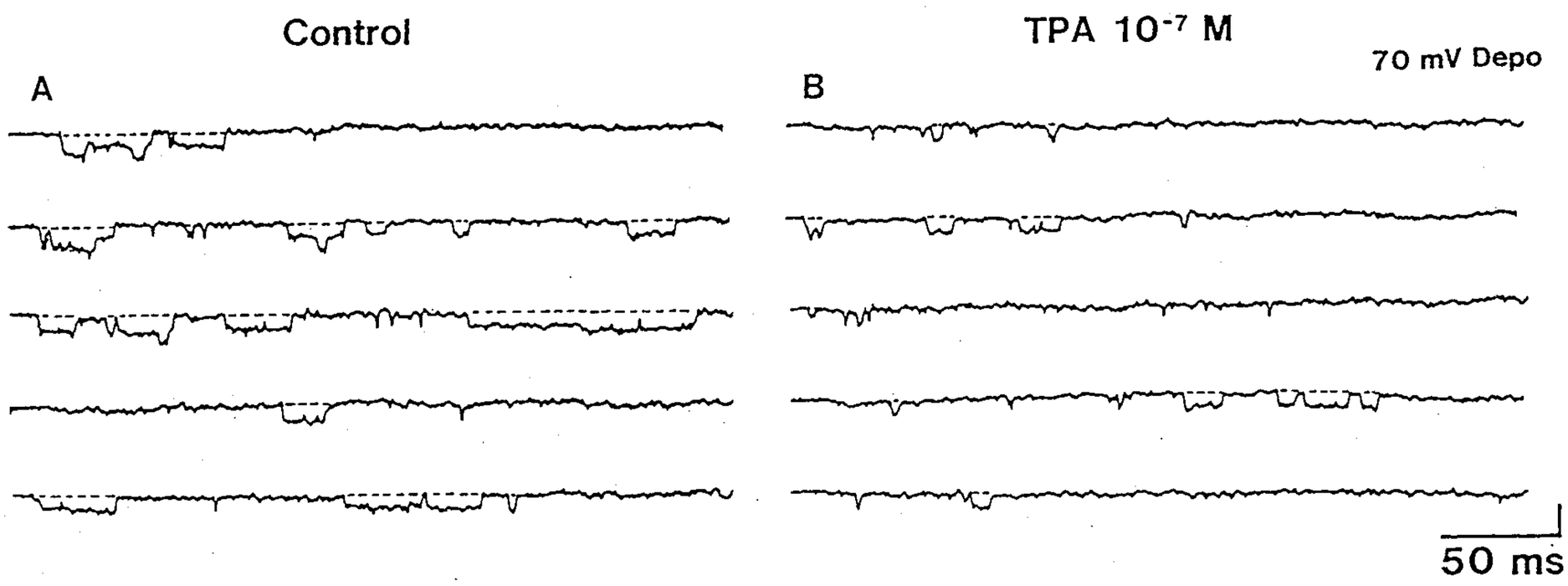

Fig. 2. Decrease in the opening of L-type Ca^{2+} channel in the presence of TPA. Cell-attached patch clamp experiments were performed using a rabbit sino-atrial nodal cardiomyocyte. Unitary channel openings are shown by the steps to the inward direction. Depolarizing test pulses of 70 mV for 300 ms were applied from the resting potential (−80 mV). **A:** Current traces of unitary L-type Ca^{2+} channel in control. **B:** Unitary current traces in TPA (10^{-7} M). Pipette solution included Ba^{2+} (50 mM) and Bay K 8644 (10^{-6} M). TTX (10^{-5} M) was added to the bath solution. Dashed line represents zero current level.

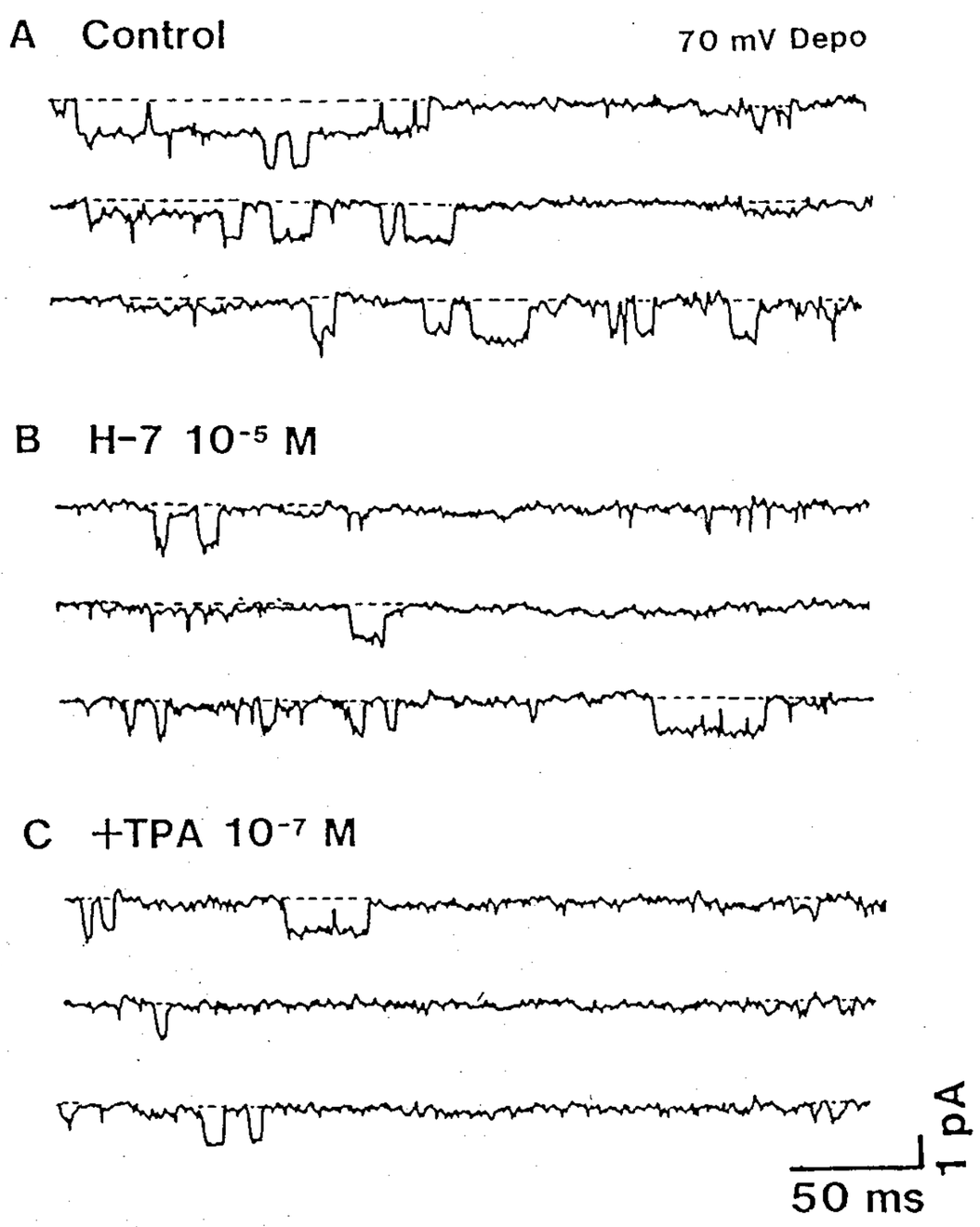

Fig. 3. Effects of H-7 and H-7 plus TPA on the L-type Ca^{2+} channel in a rabbit sino-atrial nodal cell. Cell-attached patch-clamp experiments were performed. Depolarizing test pulses of 70 mV for 300 ms were applied from the resting potential (−80 mV). **A:** Current traces of unitary L-type Ca^{2+} channel in control. **B:** Unitary current traces in H-7 (10^{-5} M). **C:** Unitary current traces in H-7 (10^{-5}) plus TPA (10^{-7}). Pipette solution included Ba^{2} (50 mM) and Bay K 8644 (10^{-6} M). TTX (10^{-5} M) was added to the bath solution. Dashed line represents zero current level.

ester analogue not activating PK-C) did not cause any effect of the channel opening, as shown in Fig. 4A and B.

Discussion

Phorbol esters not only plays a role as tumor promoters, but also produces lot of physiological responses, as mentioned above[1]. Phorbol esters substitute for DG and activate PK-C. TPA, a DG-like structure, can substitute for DG, and its primary site of action is the cell surface[19),20)]. Recent studies including our findings have showed the electrophysiological effects on the ionic channels[12),14−16)].

On the cell membrane of heart, two types (L and T) of Ca^{2+} channels are present[13),21−23)]. These are distinguished by difference in (1) their voltage-dependency of activation and inactivation, (2) their sensitivity to dihydropyridine blockers, (3) their single-channel conductance, and (4) their permeability to various divalent cations. In this study, the effects of PK-C stimulation only on the $I_{Ca(L)}$ were examined.

The effects of PK-C stimulation by phorbol

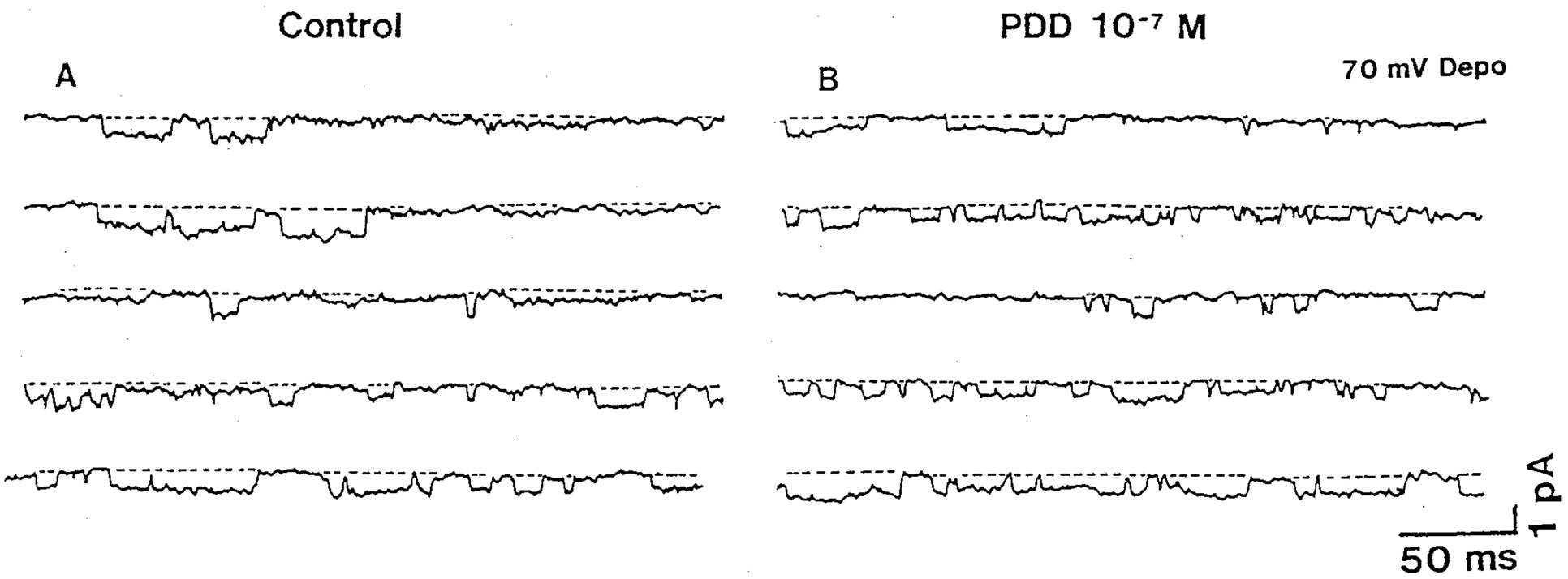

Fig. 4. Effect of PDD on the L-type Ca^{2+} channels in a sino-atrial nodal cell. Non-activator of PK-C among phorbol ester analogues, 4-alpha-phorbol-12, 13-didecanoate (PDD, 10^{-7} M), was added to the bath solution. Depolarizing test pulses of 70 mV for 300 ms were applied from the resting potential (−80 mV). **A:** Current traces of unitary L-type Ca^{2+} channel in control. **B:** Unitary current traces in PDD (10^{-7} M). Pipette solution included Ba^{2+} (50 mM) and Bay K 8644 (10^{-6} M). TTX (10^{-5} M) was added to the bath solution. Dashed line represents zero current level.

esters on the $I_{Ca(L)}$ in cardiac and vascular smooth muscle cells and in neurons are still controversial. The activation of PK-C enhanced the voltage-dependent Ca^{2+} current in bag cell neurones of the mollusc Aplysia[24], whereas TPA attenuates $I_{Ca(L)}$ in sensory neurones[25]. In rabbit SA node and neonatal rat heart, TPA slightly increased $I_{Ca(L)}$[12),26]. In smooth muscles (frog viscera and rat aorta), phorbol esters enhanced $I_{Ca(L)}$[27),28]. Recently, Satoh and Sperelakis[15),16)] in aortic vascular smooth muscles (A7r5 cell line), TPA and PDB slightly enhanced $I_{Ca(L)}$ in almost half of all cells, and inhibited $I_{Ca(L)}$ in the half cells. In the present experiments, both whole-cell and patch-clamp experiments showed that TPA inhibited the amplitude of $I_{Ca(L)}$ and the opening of L-type Ca^{2+} channels. And PDD (non-activator of PK-C) did not affect the opening. These are quite consistent with the results from guinea-pig ventricular cardiomyocytes[14]. Therefore, these results indicate that TPA inhibits $I_{Ca(L)}$ and the opening probability due to the PK-C stimulation.

H-7, a PK-C inhibitor, did not affect any significant changes in the TPA-induced depression in the Ca^{2+} channel opening, also consistent with previous reports[13),14)]. It has been known that H-7 also inhibits the different protein kinases with equal activity; cyclic nucleotides (cAMP and cGMP)-dependent kinases (PK-A and PK-G)[29]. Thus, it seems that the slight decrease in the opening of Ca^{2+} channels induced by H-7 alone may be produced by the inhibition of PK-A. I concluded that H-7 has no or little selective inhibitory action of PK-C activation.

Satoh and colleague[11−13)] have already demonstrated that phorbol esters elevate the cellular Ca^{2+} concentration ($[Ca]_i$). Because phorbol esters elicited the cellular calcium overload in spontaneously beating SA node cells and ventricular myocytes, resulting in developement of arrhythmias. In experiments using fura-2 (a fluorescent Ca^{2+} indicator), phorbol esters actually elevated $[Ca]_i$level. In the present experiments, however, TPA inhibited $I_{Ca(L)}$ and decreased the opening probability. Therefore, these results suggest that PK-C activation would elevate $[Ca]_i$ due

to other unknown intracellular functions; (1) release from Ca^{2+} stores (SR and mitochondria), (2) blockade of Ca^{2+} re-uptake into SR, (3) blockade of Ca^{2+} pump, (4) feed back of PI turnover, and (5) inhibition of Na^{+}-H^{+} or Na^{+}-Ca^{2+} exchange. Further experiments are required to elucidate this mechanism.

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プロテインキナーゼCの単一ウサギ洞房結節細胞L型 Ca^{2} チャネルに対する作用：ホールセルとセルアタッチ・パッチクランプ法による解析

佐藤　広康

奈良県立医科大学薬理学教室

要　　旨

単離したウサギ洞房結節細胞にフォルボールエステルを適用して細胞内プロテインキナーゼCを活性させ，L型 Ca^{2+} チャネル（ジハイドロピリジン感受性）への電気生理学的作用について調べた。ホールセル•クランプではTPA（10^{-7} M）は Ca^{2+} 電流を抑制し，その不活性化過程を遅延させた。セルアタッチ・クランプによる解析ではTPAは単一チャネルの開口率を著名に減少させた。チャネルコンダクタンスは18±1 pS（n＝5）のままであった。プロテインキナーゼCの抑制剤H-7（10^{-5} M）はTPAによるチャネル開口率の減少を拮抗しなかったが，PDD（プロテインキナーゼCの不活性化フォルボールエステル）は全く開口率に影響を与えなかった。H-7単独では有意差はないが，チャネル開口率を抑制した。以上の結果より，フォルボールエステルによるプロテインキナーゼCの活性化はウサギ洞房結節細胞のL型 Ca^{2+} チャネルの開口率を減少させて，Ca^{2+} 電流を抑制することが解明された。