Effects of Protein Kinase C on the Muscarinic Excitation of Rat Adrenal Chromaffin Cells

Akinori Akaike[1], Masashi Sasa[2,*], Yutaka Tamura[1], Hisamitsu Ujihara[3] and Shuji Takaori[4]

[1] *Department of Neuropharmacology, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama 729-02, Japan*
[2] *Department of Pharmacology, Hiroshima University School of Medicine, Hiroshima 734, Japan*
[3] *Department of Pharmacology, School of Medicine, Yamaguchi University, Ube 755, Japan*
[4] *Shimane Medical University, Izumo 693, Japan*

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ABSTRACT—The role of protein kinase C (PKC) in the muscarinic excitation of chromaffin cells freshly isolated from rat adrenal medullae was examined by the patch-clamp recording method. Acetylcholine and McN-A-343, a M_1-receptor agonist, depolarized the cell and induced action potentials. Phorbol 12,13-dibutyrate (PDBu), an activator of PKC, increased acetylcholine-induced firing concomitant with a persistent depolarization. Under voltage-clamp recording, both McN-A-343 and PDBu decreased the cesium-sensitive K^+ current, which was induced by shifting the membrane potential between -140 mV and -40 mV. These results suggested that the stimulation of muscarinic M_1-receptors by cholinergic drugs activated phospholipase C to degrade phosphoinositide, consequently producing diacylglycerol, and diacylglycerol activates PKC to induce excitation of adrenal chromaffin cells.

Keywords: Adrenal medulla, Muscarinic, Protein kinase C

Adrenal medullary chromaffin cells are typical paraneurons with neuron-like properties (1). Protein kinase C (PKC) is known to play an important role in the exocytotic release of catecholamine from adrenal chromaffin cells; the activation of PKC by phorbol esters causes a translocation of PKC from the intracellular fluid to the membrane to enhance catecholamine secretion (2-4). Hydrolysis of inositol-containing phospholipids in adrenal chromaffin cells and PC12 phenochromocytoma cells is triggered by stimulation of nicotinic and/or muscarinic receptors (5-7).

We have previously demonstrated that both nicotinic and muscarinic receptors mediate cholinergic excitation of chromaffin cells isolated from adult rat adrenal medullae (8, 9). The muscarinic excitation was initiated by the closing of K^+ channels which had been open at the resting membrane potential (RMP). Electrophysiological studies on sympathetic neurons have also demonstrated that PKC activators, such as phorbol esters, mimicked muscarinic receptor-mediated effects such as obtaining inward current by suppressing outward M current. This suggests that PKC works as an intracellular mediator in the muscarinic responses (10). Since muscarinic receptor-mediated excitation of adrenal chromaffin cells shows characteristics similar to those observed in sympathetic neurons (8-10), the excitation is probably produced by phosphorylation-induced closure of potassium channels due to activation of PKC. Therefore, the present study was performed by patch-clamp recording on acutely isolated chromaffin cells to examine this possibility.

The procedures for obtaining acutely dissociated adrenal chromaffin cells were similar to those described previously (8). Briefly, adrenal medullae obtained from male Wistar rats (150-250 g) were incubated for 30-35 min at 33-34℃ in low Ca^{2+} medium containing collagenase (0.3%), hyaluronidase (0.1%) and trypsin (0.1%). Single chromaffin cells were separated by agitation with a stream of medium and were perfused with HEPES-buffered solution which consisted of: 116.4 mM NaCl, 5.4 mM KCl, 1.3 mM $MgSO_4$, 3.0 mM HEPES, 1.5 mM $CaCl_2$, 26.2 mM $NaHCO_3$ and 11.0 mM glucose, (pH 7.2). The superfusion fluid was maintained between 31℃ and 32℃ and continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2.

* To whom correspondence should be addressed.

The patch microelectrodes were prepared according to the technique described by Hamill et al. (11). Spontaneous firings of chromaffin cells were extracellularly recorded after formation of the giga-ohm seal in a cell-attached configuration. The pipette solution for extracellular recordings had the same composition as that used for the superfusion fluid. The standard pipette solution used in the whole-cell recordings consisted of: 140.0 mM KCl, 2.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 11.0 mM ethylenglycol-bis (b-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 10.0 mM HEPES. The pH of the solution was adjusted to 7.2 by adding a small amount of KOH. Current-clamp and voltage-clamp recordings were made with an EPC-7 patch-clamp system (List Electronic, Germany). The signals were monitored on a digital oscilloscope (VC-10, Nihon Kohden, Tokyo) and simultaneously stored on an FM data recorder (A-65, Sony, Tokyo) for later analysis.

For fast application of the drugs, the drug-containing solution filled in a glass pipette with an inner diameter of 100–120 μm was directly perfused onto the cell which was recorded from a distance of approximately 100 μm using a motor-drive microinjector (IM-1, Narishige, Tokyo) following the method described in the previous paper (8).

Drugs used were acetylcholine (ACh) chloride, McN-A-343 and phorbol-12,13-dibutyrate (PDBu) (Sigma, U.S.A.). ACh and McN-A-343 were dissolved in the perfusing medium. PDBu was first dissolved in ethanol to yield a concentration of 10^{-3} g/ml and then kept below −10℃ as a stock solution. The stock solution of PDBu was then diluted in the perfusing medium to obtain the final concentration.

The freshly dissociated chromaffin cell showed firing with a very low frequency of less than 1 Hz. In the extracellular recording, application of ACh at 10^{-5} M induced firing in 5 of 7 cells tested, and McN-A-343, a selective M_1-receptor agonist, at 10^{-5} M also induced spike generation in 5 of 6 cells tested (Fig. 1). In the whole-cell recording, ACh at 10^{-5} M depolarized the cell with a rapid rising phase at the onset of the effect followed by a slow rising phase. McN-A-343 at 10^{-5} M also depolarized the cell with a slow rising phase and a decay phase. Both drugs induced firing of the recorded cell when drug-induced depolarization was large enough to exceed the threshold of an action potential.

In the extracellular recording, PDBu at 10^{-8} and 10^{-7} M did not induce firing in any of the 4 or 5 cells, respectively, that were examined. However, when ACh at 10^{-5} M was added to the bath to increase the firing rate of the cells and then PDBu at 10^{-7} M was applied, ACh-induced firing was further increased in all 5 cells examined (Fig. 2). In addition, in the whole-cell recording, PDBu at 10^{-8} and 10^{-7} M dose-dependently induced depolarization, which lasted more than one min after the cessation of the application in all 10 cells examined. Depolarization induced by PDBu (10^{-7} M) was 13.5±2.4 mV (mean± S.E.), which was not large enough to elicit action potentials.

The effects of the drugs on the steady state current/voltage (I/V) relation were examined by passing a slow

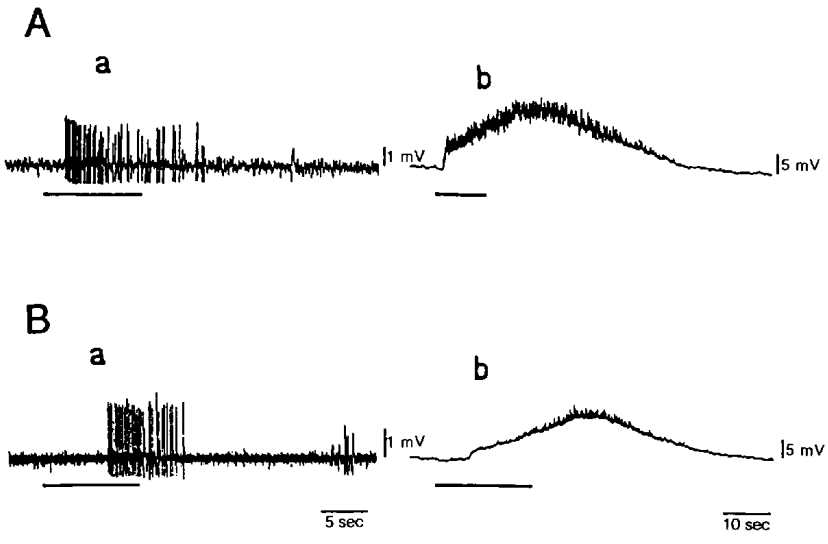

Fig. 1. Effects of ACh (10^{-5} M, A) and McN-A-343 (10^{-5} M, B) on the activity of adrenal chromaffin cells recorded either as extracellular (A-a and B-a) or as whole-cell recordings (A-b and B-b). Bars under the recording traces show the period of drug application (5–10 sec).

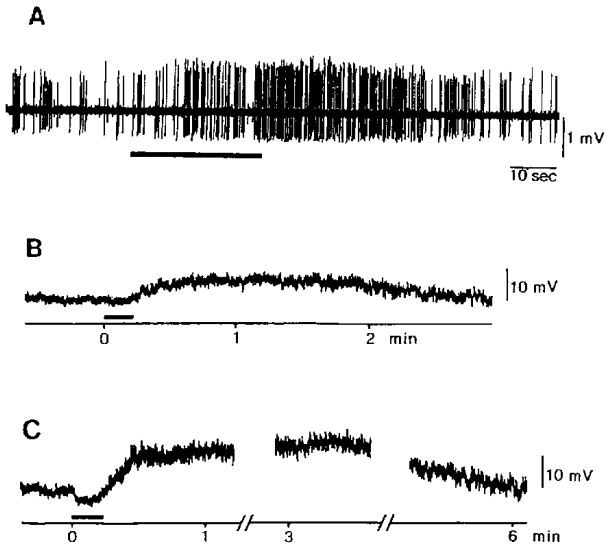

Fig. 2. Effects of PDBu on the activity of adrenal chromaffin cells. A: effects of PDBu (10^{-7} M) on ACh-induced firing in extracellular recording. ACh (10^{-5} M) was continuously added to the bath to induce spontaneous activity of the cell. B and C: effects of PDBu at 10^{-8} (B) and 10^{-7} M (C) in whole-cell recording. Bars under the recording traces show the period of drug application.

(100 mV/sec) ramp voltage command and measuring the resulting membrane current (Fig. 3). The current induced by the ramp voltage command was reduced by the addition of cesium (2×10^{-3} M) to the outside of the cell. The blockade of the current by cesium was evident when the membrane potential was clamped between -140 mV and -80 mV. Partial recovery of cesium-induced reduction of the current was observed 5 min after cessation of the application. McN-A-343 at 10^{-5} M and PDBu at 10^{-7} M also reduced the current induced by the ramp command pulse in all 4 cells examined. Reduction of the current by McN-A-343 or PDBu usually recovered to the control level 5 min after cessation of the application.

The present study showed that activation of PKC by PDBu produces depolarization and enhances ACh-induced excitation. Both PDBu and an M_1-agonist, McN-A-343, reduced the current induced by the ramp voltage command. Since the current induced by the ramp command pulse of between -140 and -40 mV was reduced by the application of cesium to the outside of the membrane, the major component of the current is considered to be a K^+ current (12). Therefore, it is suggested that both PDBu and McN-A-343 reduced K^+ conductance of the adrenal chromaffin cells at the potentials near the RMP, resulting in depolarization which increases the excitability of the cell. The biochemical studies using PC12 pheochromocytoma cells have shown that muscarinic receptor stimulation increases diacylglycerol concentration either by increasing phosphoinositide hydrolysis (13) or by sources other than inositol-containing phospholipids (14). Therefore, our present results, together with these findings, suggest that M_1-receptor stimulation generates diacylglycerol which activates PKC, resulting in the induction of phosphorylation of K^+ channels, which reduces K^+ conductance at potentials near the RMP.

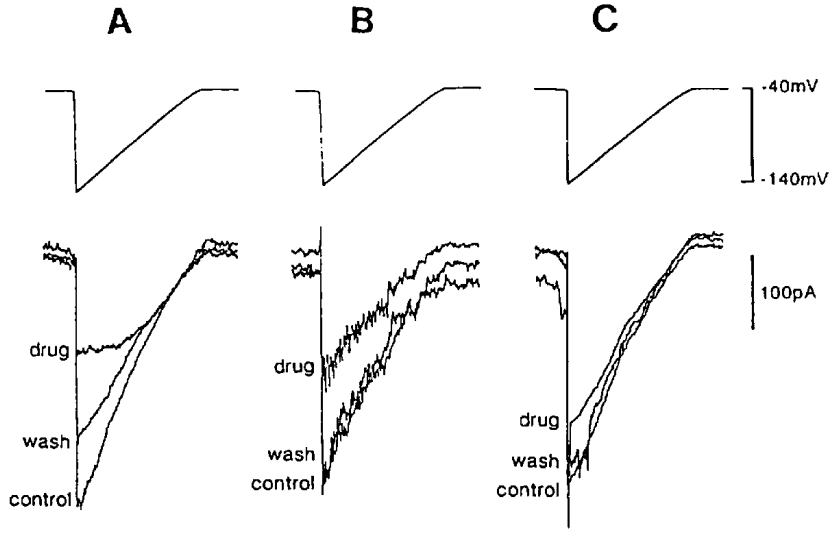

Fig. 3. Effects of cesium (2×10^{-3} M, A), McN-A-343 (10^{-5} M, B) and PDBu (10^{-7} M, C) on the currents induced by a ramp voltage command which was given under voltage-clamp recording. Upper and lower traces show the changes of membrane potential and membrane current, respectively.

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