Effect of forskolin on acetylcholine-induced current in rat pheochromocytoma cells

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KEY WORDS forskolin；nicotinic receptors；PC12 cells；cyclic AMP；adenylate cyclase；patch-clamp techniques；8-bromo cyclic adenosine monophosphate；cyclic AMP-dependent protein kinase

ABSTRACT

AIM：To study the effect of forskolin on the nicotinic receptor (NicR) of PC12 cells. METHODS：The acetylcholine (ACh)-induced current (I_{ACh}) was measured on PC12 cells by whole-cell clamp technique. RESULTS：The I_{ACh} could be blocked by *d*-tubocurarine chloride and atropine had no effect on I_{ACh}. Infusion of forskolin (1 – 50 μmol·L^{-1}) caused an inhibition on I_{ACh}, which was reversible, concentration-dependent, and voltage-independent. Preincubation with 8-bromo-adenosine-3′, 5′-adenosine monophosphate (8-Br-cAMP), a cell-permeable cAMP analog which preferentially activated cyclic AMP-dependent protein kinase (CADPK), for 20 min, did not affect the I_{ACh} and the inhibitory effect of forskolin. Infusion of 1,9-dideoxyforskolin, an analog of forskolin which did not activate adenyl cyclase, also caused an inhibition on I_{ACh}. CONCLUSION：The inhibitory effect of forskolin on I_{ACh} in PC12 cells is not mediated by activating the adenyl cyclase. Probably, the lipophilic forskolin acts via perturbing the plasma membrane lipid structure and altering the function of the NicR.

INTRODUCTION

Nicotinic receptor (NicR) is a ligand-gated ion channel, and it is one of the earliest examples of ion channel modulated by phosphorylation. Early experiments demonstrated that the *Torpedo* NicR was subjected to cAMP-dependent phosphorylation and was regulated by phosphorylation[1]. When this possibility was investigated with neuronal NicR of pheochromocytoma (PC12) cells, no evidence could be obtained to support the hypothesis that acute elevation of cAMP can alter the ion-conducting properties of the neuronal NicR[2]. However, in our previous studies, we observed that forskolin (1 – 50 μmol·L^{-1}), a powerful activator of adenylate cyclase, caused a rapid inhibition on I_{ACh} in PC12 cells. In the present work, we further examined the effect of acute treatment of PC12 cells with forskolin and found that the inhibition by forskolin was not mediated by cAMP.

MATERIALS AND METHODS

Cell culture PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10 % fetal bovine serum and 5 % horse serum (Gibco). Cells were plated onto collagen-coated cover-slips which were placed in 35-mm plastic tissue culture dishes and treated with 2.5 S-nerve growth factor (2.5 S-NGF, Sigma) at 50 μg·L^{-1}. The cultures were fed three times a week and were used for electrophysiological experiment 5 – 10 d after being plated.

Whole cell patch-clamp recording Cover-slips bearing PC12 cells were removed from culture medium and placed into the recording chamber (volume approximately 0.6 mL) which was perfused continuously with extracellular solution containing NaCl 140, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1, HEPES 10, glucose 11.5 mmol·L^{-1}, pH 7.4. The basic patch electrode-filling solution included KCl 140, egtazic acid 0.5, HEPES 5, $MgCl_2$ 1, $CaCl_2$ 0.397, NaOH 3, ATP-2Na 1 mmol·L^{-1}, pH 7.2 – 7.3. Recording were obtained from isolated cells lacking processes using standard patch-clamp techniques[3]. Patch-recording electrodes were pulled from borosilicate glass micropipettes on a PC-10 puller (Narishige, Japan). Electrode resistance in recording solution was 2 – 5 MΩ.

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Membrane currents from whole-cell voltage clamp were recorded using a CEZ-2300 amplifier (Nihon Kohden, Japan) and were fed to a pen recorder (LMS-2B, Chengdu, China, frequency response 5 Hz at − 3 dB). Chemicals were diluted in the extracelullar perfusing solution. External solutions were changed by gravity feed and flow was controlled via three-way valves fixed in proximity to the bath. The solution changing time was minimized to 3 − 8 s. Cells were voltage-clamped to − 80 mV unless otherwise stated. All experiments were performed at room temperature (22 − 24 ℃).

Statistical analysis Data were expressed as $\bar{x} \pm s$. Paired t test was used for testing the significance between the peak amplitude of I_{ACh} before and after perfusion of the test drug. ANOVA was used for testing the significance among the inhibitory rates of forskolin at various concentrations or holding potentials.

RESULTS

I_{ACh} in PC12 cells When cultured in the presence of NGF, PC12 cells differentiate to resemble sympathetic neurons morphologically, accompanied by physiological and biochemical changes which ultimately endow them with a neuron-like function[4]. After the membrane was ruptured, the membrane potential was held at − 80 mV. Bath application of ACh 30 μmol·L^{-1} produced an inward current associated with an increase in current noise, a reflection of stochastic behavior of the activated channels. The peak amplitude of I_{ACh} was (0.62 ± 0.11) nA (n = 24). When ACh was continuously present in the bath for 40 s, the current amplitude gradually decreased to a sustained level due to the desensitization process (Fig 1).

Application of nicotine (20 μmol · L^{-1}) also produced an inward current similar to I_{ACh}. The I_{ACh} was blocked by simultaneous application of *d*-tubocurarine chloride (100 μmol · L^{-1}), which was a blocker of nicotinic receptor. However, atropine (10 μmol·L^{-1}), as a blocker of muscarinic receptor, had no effect on I_{ACh} (data not shown). Thus we assumed that the I_{ACh} was generated through the NicR, which was in agreement with the report of Boyd[5].

Rapid inhibition by forskolin on I_{ACh} in PC12 cells When voltage-clamped at − 80 mV, cells were pretreated with forskolin (0.5 − 50 μmol·L^{-1}) for 2 min, then ACh (30 μmol · L^{-1}) was applied simultaneously with the corresponding concentration of forskolin. The peak amplitude of I_{ACh} was greatly inhibited. The analysis of variance showed that there was significant

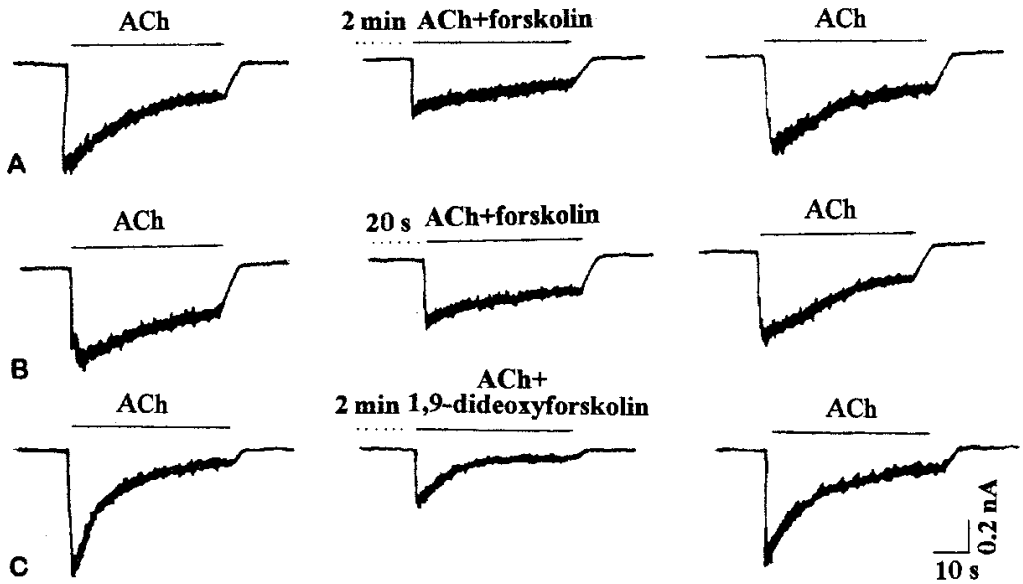

Fig 1. Inhibition of I_{ACh} in a PC12 cell by extracellular application of forskolin or 1,9-dideoxyforskolin. Pretreatment with forskolin for 2 min (A) or 20 s (B); Pretreatment with 1,9-dideoxyforskolin for 2 min (C). Simultaneous application of forskolin (or 1,9-dideoxyforskolin) and ACh. Periods of applications are indicated by bars. Holding potential: − 80 mV. ACh 30 **μmol·L^{-1}**, **forskolin** 10 **μmol·L^{-1}**, **1,9-dideoxyforskolin** 10 **μmol·L^{-1}**.

difference among the rates of inhibition ($F = 38.6$, $P < 0.01$) (Fig 1A and Tab 1). Then analysis of correlation was performed between the concentration of forskolin and the rate of inhibition. Correlation coefficient (r) was 0.9, which indicated that the inhibitory effect of forskolin on I_{ACh} was concentration-dependent. This inhibition by forskolin was reversible and was easily eliminated by its washout. When the pretreatment time of forskolin was shortened to 20 s, the inhibition on I_{ACh} was also very apparent (Fig 1B). The cAMP level was still at basal levels in such a short period[6]. This result indicated that the inhibitory effect of forskolin on I_{ACh} occurred move rapidly than on cellular cAMP levels.

Tab 1. Inhibitory effect of perfusion of forskolin on I_{ACh} in PC12 cells. $n = 6$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* amplitude of I_{ACh} before pretreatment with forskolin.

Forskolin/ μmol·L^{-1}	I_{ACh}/pA Before perfusion	I_{ACh}/pA After perfusion	Rate of inhibition/ %
0.5	612 ± 104	610 ± 65	0.3 ± 0.2
1	618 ± 59	545 ± 53^b	11.8 ± 0.4
5	611 ± 69	321 ± 27^c	47.4 ± 5.8
10	627 ± 107	314 ± 19^c	50.0 ± 4.7
50	609 ± 101	207 ± 28^c	66.1 ± 3.8

The cells were incubated with different concentrations of forskolin for 2 min, and then were given the same corresponding concentration of forskolin and ACh 30 μmol·L^{-1} simultaneously. I_{ACh} (nA): peak amplitude of I_{ACh}. Before perfusion, After perfusion: Before and after perfusion of forskolin.

To determine the influence of the membrane potential on the inhibition by forskolin on I_{ACh}, we firstly recorded the current-voltage relationship of responses to ACh in control conditions and then in the presence of forskolin 10 μmol·L^{-1} after pretreating cells for 2 min. It could be seen from Fig 2 that forskolin had rapid inhibition on I_{ACh} at various holding potentials from −90 to −40 mV, and there was significant difference between the peak currents before and after pretreatment with forskolin. However, analysis of variance showed no significant differences among the rates of the inhibition ($P > 0.05$). These results indicate that the inhibition by forskolin on I_{ACh} is voltage-independent.

Effect of 8-Br-cAMP on I_{ACh} in PC12 cells

8-Br-cAMP is a cell-permeable cAMP analogue, and it preferentially activates CADPK[7]. Hei *et al* have reported that when rat vas deferens were incubated with 8-Br-cAMP 1 μmol·L^{-1}, CADPK was shown to be

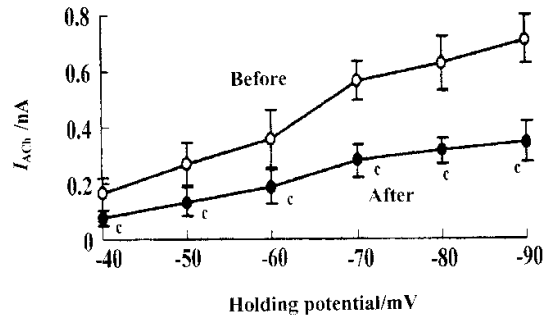

Fig 2. Inhibition by forskolin on I_{ACh} in PC12 cells at various holding potentials. The cells were incubated with forskolin (10 μmol·L^{-1}) for 2 min at various holding potentials, and then were applied with forskolin (10 μmol·L^{-1}) and ACh 30 μmol·L^{-1} simultaneously. (○, ●): Before and after pretreatment with forskolin for 2 min. $n = 6$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* the peak amplitude of I_{ACh} before pretreatment with forskolin.

remarkably activated after 1 min. In the present work, PC12 cells were incubated in an extracellular solution containing 8-Br-cAMP (10 – 100 μmol·L^{-1}) for 20 min. After the membrane was ruptured, the I_{ACh} was recorded. The peak amplitude of I_{ACh} (0.61 ± 0.13, $n = 6$) was neither increased nor decreased in comparison with that of I_{ACh} perfused with normal extracellular solution. This result indicated that 8-Br-cAMP had no effect on I_{AC} although it could activate CADPK. When the cells were extracellularly pretreated with forskolin 10 μmol·L^{-1} for 2 min in the presence of 8-Br-cAMP after incubation with 8-Br-cAMP for 20 min, the inhibitory effect of forskolin still existed and was not influenced. The inhibitory rate was 51% ± 4% ($n = 6$). The inhibition disappeared when forskolin was washed out.

Effect of 1,9-dideoxyforskolin on I_{ACh} in PC12 cells

To determine whether the effect of forskolin on I_{ACh} was a cAMP-mediated effect or a direct effect, we performed similar experiments with 1,9-dideoxyforskolin, an analog of forskolin which does not activate adenyl cyclase[8]. In contrast to forskolin 10 μmol·L^{-1}, which elevated cAMP levels in PC12 cells approximately 80-fold, 1,9-dideoxyforskolin 10 μmol·L^{-1} did not greatly affect the cAMP level of PC12 cells[6]. However, 1,9-dideoxyforskolin did inhibit I_{ACh} in a concentration-dependent manner. At various concentrations of 1,9-dideoxyforskolin (0.5, 1, 5, 10, 50 μmol·L^{-1}), the inhibitory rates on peak I_{ACh} were 13 % ± 3 %, 30 % ± 4 %, 51 % ± 6 %, 65 % ± 4 %, 73 % ± 3 % ($n = 6$), respectively. The inhibition induced by

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1，9-dideoxyforskolin appeared to be even more potent than forskolin in the corresponding concentration. It was also rapidly reversible (Fig 1C). These results strongly suggest that the effect of forskolin on I_{ACh} is a direct effect rather than a cAMP-mediated effect.

DISCUSSION

It is known that phosphorylation plays an important role in desensitization of NicR and the phosphorylation of this receptor of *Torpedo* and neuromuscular junction is catalyzed by cAMP-dependent protein kinase[1]. However, acute elevation of cAMP can not alter the ion-conducting properties of the neuronal NicR[2]. In the present experiment, it seems that I_{ACh} is directly inhibited by either forskolin ($1 - 50\ \mu mol \cdot L^{-1}$) or 1，9-dideoxyforskolin ($0.5 - 50\ \mu mol \cdot L^{-1}$). Although pretreating cells with forskolin ($10\ \mu mol \cdot L^{-1}$) for 20 s could not increase the cellular cAMP content[2], it could induce a rapid inhibition on I_{ACh} in PC12 cells. As an analog of forskolin, 1，9-dideoxyforskolin did not activate adenyl cyclase, but it did inhibit I_{ACh} in a concentration-dependent manner and it appeared to be even more potent than forskolin. These results suggest that the effect of forskolin is independent of cellular cAMP changes. Further evidence supporting this notion emerged from the experiment of 8-Br-cAMP, which exerts its effect as an activator of CADPK. 8-Br-cAMP ($10 - 100\ mol \cdot L^{-1}$) did not affect either I_{ACh} or the inhibition on I_{ACh} by forskolin. The inhibitory effect of forskolin on I_{ACh} was concentration-dependent and voltage-independent. The latter excluded the possibility that forskolin acted as an open channel blocker (OCB). In general, OCBs are strongly voltage-dependent, due to the charge they carry in the transmembrane field[9].

Lorenzon *et al* have studied the action of forskolin on NicR channels on cultured rat muscle fibres in the cell-attached configuration. Their work provided evidence that the channel activity of muscle embryonic-type NicR could be influenced by a direct action of forskolin, and was also greatly reduced by an indirectly-mediated cytosolic mechanism triggered by forskolin[10]. In the present study, we investigated the effect of forskolin on the neuronal NicR in PC12 cells. We do not know exactly whether the mechanism of the effect of forskolin on neuronal NicR is the same as on muscle embryonic-type NicR. The concentrations of forskolin in our studies were lower than those in Lorenz's experiments. Our results suggest that forskolin ($1 - 50\ \mu mol \cdot L^{-1}$) can inhibit I_{ACh} in PC12 cells and the effect is independent of cellular cAMP. Although forskolin exerts its effect primarily by directly activating the catalytic unit of adenyl cyclase[8], at high concentrations ($1\ mmol \cdot L^{-1}$) it has been observed to perturb lipid organization in rat liver plasma membrane[11]. Considering the very lipophilic nature of the forskolin molecule, we considered the most likely explanation for our results is that forskolin is entering the plasma membranes and is disrupting the lipid structure. The observation of a slightly greater potency for 1，9-dideoxyforskolin than forskolin also would be consistent with lipid perturbation. Removal of two hydroxyl groups would produce a lipophilic molecule which would distribute into membranes more readily.

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弗司扣林对大鼠肾上腺嗜铬细胞瘤细胞乙酰胆碱诱发电流的影响

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关键词 弗司扣林；烟碱受体；PC12 细胞；环腺苷一磷酸；腺苷酸环化酶；膜片箝技术；8-溴环腺苷一磷酸；cAMP 依赖性蛋白激酶

目的：研究弗司扣林对 PC12 细胞烟碱受体的作用及其机制. 方法：采用全细胞膜片箝技术记录 PC12 细胞乙酰胆碱诱发电流（I_{ACh}）. 结果：PC12 细胞上乙酰胆碱诱发电流是由烟碱受体引起的. 弗司扣林 1－50 μmol·L^{-1}可快速抑制 I_{ACh}，此作用呈可逆性、浓度依赖性和非电压依赖性. 用可透过细胞膜的 cAMP 类似物 8-溴-环单磷酸腺苷（8-Br-cAMP）孵育细胞 20 min，既不影响 I_{ACh}，也不影响弗司扣林对 I_{ACh} 的抑制作用. 弗司扣林的类似物 1，9-dideoxyforskolin 不能激活腺苷酸环化酶，但也能快速抑制 I_{ACh}. 结论：弗司扣林对 PC12 细胞 I_{ACh}的快速抑制作用不是由腺苷酸环化酶的活化介导的，而很可能是由其对膜脂质结构的扰动作用从而改变烟碱受体的功能引起的.

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