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European Journal of Pharmacology 337 (1997) 95–102

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Phosphodiesterase type 4 that regulates cAMP level in cortical neurons shows high sensitivity to rolipram

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Received 22 July 1997; revised 15 August 1997; accepted 19 August 1997

Abstract

To characterize the role of phosphodiesterase type 4 (a cAMP-specific and rolipram-sensitive phosphodiesterase) among phosphodiesterases in the regulation of the intracellular cAMP level in cortical neurons, we investigated the effects of phosphodiesterase inhibitors on the intracellular cAMP levels in primary cultured rat cortical neurons. Selective inhibitors of phosphodiesterase type 4 and type 2 significantly enhanced β-adrenoceptor-mediated cAMP increase. Selective inhibitors of phosphodiesterase type 1, type 3 and type 5/6 had no effect on the cAMP level. Rolipram enhanced the β-adrenoceptor-mediated cAMP increase in cortical neurons, astrocytes and vascular smooth muscle cells at different minimum effective concentrations (10, 100 and 1000 nM, respectively). These findings indicate that phosphodiesterase type 4, showing a high-sensitivity to rolipram, plays a major role in regulating cAMP in the cortical neurons, and that rolipram at low doses enhances the intracellular cAMP increase in the cortical neurons selectively. © 1997 Elsevier Science B.V.

Keywords: Phosphodiesterase; Rolipram; Cortical neuron; cAMP

1. Introduction

A number of neuronal functions are known to be controlled by intracellular cAMP and cAMP-dependent protein kinase, e.g., release of noradrenaline and dopamine from rat brain slices (Schoffelmeer et al., 1985), excitation of locus coeruleus neurons (Wang and Aghajanian, 1987), activity of tyrosine hydroxylase (Kaufman, 1995), neurite-outgrowth (Mattson et al., 1988), conductance of ion channels (Hell et al., 1995; Moreno et al., 1995), survival of dopaminergic neurons (Michel and Agid, 1996), etc. Intracellular cAMP is generated in response to activation of adenylyl cyclase and is degraded by cyclic nucleotide phosphodiesterase, the enzyme that catalyzes the hydrolysis of cAMP to 5′-AMP (Beavo, 1995). Thus, inhibition of phosphodiesterase enhances intracellular cAMP accumulation, which would lead to potentiation of cellular functions mediated by cAMP. Seven different types of phosphodiesterases (type 1 to 7) are known to be present in mammalian tissues (Beavo, 1995). Phosphodiesterase type 4 and type 7 are cAMP-specific phosphodiesterases, whereas phosphodiesterase type 1, type 2 and type 3 hydrolyze both cAMP and cGMP and phosphodiesterase type 5 and type 6 are cGMP-specific phosphodiesterases. Four types of phosphodiesterase, type 1, type 2, type 3 and type 4 have been demonstrated to be present in the brain (Beavo, 1995; Reinhardt and Bondy, 1996). Phosphodiesterase type 4 is characterized by its specific sensitivity to rolipram, a selective phosphodiesterase type 4 inhibitor and exists in a variety of organs including the brain and vascular tissues (Stoclet et al., 1995). Recently, many phosphodiesterase type 4 isozymes have been found, and these isozymes show different sensitivities for rolipram whose binding sites are regarded to be associated with phosphodiesterase type 4 (Müller et al., 1996). However, cellular distributions and functions of the phosphodiesterase type 4 isozymes in the brain remain unclear, although there is some information about their expression (Lobban et al., 1994; McPhee et al., 1995).

Rolipram shows some neurotropic effects when given systemically, e.g., an antidepressive effect (Fleischhacker et al., 1992), induction of choline acetyltransferase activity in aged rat brains (Asanuma et al., 1993) and amelioration of learning and memory impairments induced by brain-ischemia in rats (Imanishi et al., 1997). These findings indicate that phosphodiesterase type 4 plays an important role in the central nervous system, and rolipram may be effective to increase the intracellular cAMP levels in brain

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cells. Rolipram inhibits phosphodiesterase type 4 in cardiovascular tissues and enhances the effects of cAMP-elevating drugs on cardiac contractility (Muller et al., 1990) and vascular smooth muscle relaxation (Komas et al., 1991). As for clinical pharmacology, any effect on the cardiovascular system is important in determining severe side-effects, so a comparitive study of cellular sensitivity to rolipram in brain neurons and vascular smooth muscle cells will give valuable information on phosphodiesterase type 4 functions and the effects of its inhibitors.

To study the functional role of phosphodiesterase type 4 in cortical neurons, we compared the effects of selective phosphodiesterase inhibitors on the intracellular cAMP level in primary cultured rat cortical neurons with or without β-adrenoceptor stimulation. We also compared the effects of rolipram on cAMP levels in neurons, astrocytes and vascular smooth muscle cells.

2. Materials and methods

2.1. Primary culture of rat cortical neurons

15- or 16-day-old embryos of Wistar rats were obtained from pregnant rats (Japan SLC) anesthetized with ether and the cerebral cortices of the embryos were dissected in L-15 medium (Gibco) and incubated at 37°C for 30 min in Dulbecco's phosphate-buffered saline (PBS; Nissui) containing 0.25% trypsin (Difco) and 0.5% glucose. The tissues were triturated in Dulbecco's modified Eagle's medium (DME; Nissui) containing 1% glucose and 10% fetal bovine serum (Gibco). The dissociated cells were seeded onto poly-L-lysine-coated 96-well culture plates (Sumitomo) at a plating density of 3×10^5 cells/cm^2 and incubated at 37°C in a humidified 5% CO_2–95% air atmosphere. The culture medium was changed into serum-free DME containing 1 g/l bovine serum albumin, 5 mg/l insulin, 5 mg/l transferrin, 5 μg/l sodium selenite, 100 μM putrescine and 20 nM progesterone, 24 h after the plating. Half of the medium in each well was changed twice a week and the neurons were cultured for 15–16 days and used for experiments.

2.2. Culture of rat cortical astrocytes

Rat cortical astrocytes were cultured as previously described (Yamashita et al., 1992). In brief, the cerebral cortices were dissected from 3-day-old Wistar rats (Japan SLC) and dissociated by means of trypsinization and pipetting. The cells were collected by centrifugation and resuspended in DME containing 10% fetal bovine serum, plated in 75 cm^2 culture flasks and incubated at 37°C under 5% CO_2. The cells were cultured for 3 weeks and subcultured at a plating density of 4×10^4 cells/cm^2 in 96-well culture plates for another 2 weeks. An astrocyte monolayer was formed in every culture well and used for experiments.

2.3. Culture of human vascular smooth muscle cells

Commercially available human vascular smooth muscle cells (the third subcultured cells, Kurabou) were cultured at 1×10^5 cells/cm^2 in 96-well culture plates at 37°C under 5% CO_2 in a commercially available low-serum culture medium for these cells (Kurabou). The medium was changed every 2 days and the cells were used for experiments after 10 days' culture.

2.4. Incubation of the cells with phosphodiesterase inhibitors and / or an activator of adenylyl cyclase or guanylyl cyclase

The cultured cells were washed with a balanced salt solution (BSS; 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.3 mM $MgSO_4$, 10 mM glucose and 20 mM Hepes, pH 7.4) and incubated at room temperature with BSS containing phosphodiesterase inhibitors and/or activators of adenylyl cyclase or guanylyl cyclase in the following procedures. To determine the change in intracellular cAMP in response to β-adrenoceptor activation, the cells were incubated with BSS containing 10 and 100 nM isoproterenol (Sigma) for 0–30 min. The following selective phosphodiesterase inhibitors were used at concentrations 3–5 times higher than the reported values of K_i or IC_{50} (Beavo, 1995; Stoclet et al., 1995) to selectively and sufficiently inhibit each isozyme of phosphodiesterase: 60 μM vinpocetine (a phosphodiesterase type 1 inhibitor, Calbiochem), 15 μM erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA, a phosphodiesterase type 2 inhibitor, Sigma), 1 μM milrinone (a phosphodiesterase type 3 inhibitor, Sigma), 1 μM rolipram (a phosphodiesterase type 4 inhibitor, Schering), 10 μM (butoxy-4-methoxybenzyl)-2-imidazolilinone (Ro 20-1724, a phosphodiesterase type 4 inhibitor, Bio Mol) and 1.5 μM zaprinast (phosphodiesterase type 5 and phosphodiesterase type 6 inhibitor, Sigma). To investigate the effects of these phosphodiesterase inhibitors on intracellular cAMP and cGMP levels in the cortical neurons, the cells were incubated with BSS containing each phosphodiesterase inhibitor or vehicle for 90 min, followed by incubation with BSS including 100 nM isoproterenol or 10 μM 3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine (NONOate, Cayman), a guanylyl cyclase stimulant, in addition to each phosphodiesterase inhibitor for 30 min. In some experiments, the cells were incubated with BSS containing isoproterenol or each phosphodiesterase alone to investigate the effect of each phosphodiesterase inhibitor on the cAMP and cGMP levels in the cortical neurons without stimulants. To compare the effect of rolipram on the intracellular cAMP levels in cortical neurons, astrocytes and vascular smooth muscle cells, these cells were incubated with BSS containing rolipram (1, 10, 100 and 1000 nM) or with vehicle for 90 min followed by incubation with BSS including 100 nM isoproterenol and rolipram for 30 min.

In all experiments, the reaction was terminated by removing the supernatants and adding a mixture of 0.1 M $HClO_4$ and 0.03 M EDTA. The cells were stored at −80°C.

2.5. Enzyme immunoassay

The cells were sonicated and centrifuged at 7500 × *g* for 15 min. The pellets were used to determine the protein content in each well using a protein assay reagent (Pierce). Cyclic AMP in the supernatants was measured by an enzyme immunoassay with a partial modification of a procedure described previously (Linden et al., 1992). In brief, samples and standard cAMP were acetylated with a mixture of acetic anhydride and triethylamine (1:1) and incubated with in-house rabbit anti-cAMP antiserum in immunoplates (Nunc) which were coated with an anti-rabbit immunoglobulin type G (IgG) antibody (Dako) at 5°C for 120 min. Then, conjugate of cAMP and peroxidase was added to each well and incubated at 5°C for another 60 min. Each well in the plate was washed with 0.01 M phosphate buffer containing 0.05% Tween 20. The amount of peroxidase-labeled cAMP bound to the antiserum was determined by adding 3,3′,5,5′-tetramethylbendizine/hydrogen peroxide single-pot substrate (Kierkegaard & Perry Lab). The reaction was stopped by the addition of 1 M phosphate and the absorbance at 450 nm was measured using a microtitre plate spectrophotometer (Bio-Rad). In some experiments, cGMP in the supernatants was measured using a commercially available enzyme immunoassay kit (Amersham). Intracellular cAMP and cGMP levels were expressed as fmol/μg protein or percentages of the mean in the untreated groups.

2.6. Statistics

In each experiment, the significance of differences between groups treated with vehicle (the untreated group) and with each activator alone (the control or positive-control group) was determined by means of Student's or Aspin–Welch's *t*-test. The significance of intergroup differences was determined by applying Dunnett's multiple comparison test.

3. Results

3.1. Effects of isoproterenol on the intracellular cAMP levels in cultured cortical neurons, astrocytes and vascular smooth muscle cells

The intracellular cAMP level in cortical neurons exposed to a β-adrenoceptor agonist, isoproterenol, increased rapidly and reached a peak (190 and 390% of basal levels at 10 and 100 nM, respectively) within 5 min (Fig. 1A). Then, the cAMP level decreased gradually to the baseline level but remained substantially higher than the baseline for at least 30 min. The intracellular cAMP levels in astrocytes showed a rapid increase in response to isoproterenol and reached a peak at 10 min, then stayed a high level for at least 30 min (Fig. 1B). The intracellular cAMP levels in vascular smooth muscle cells exposed to 100 nM

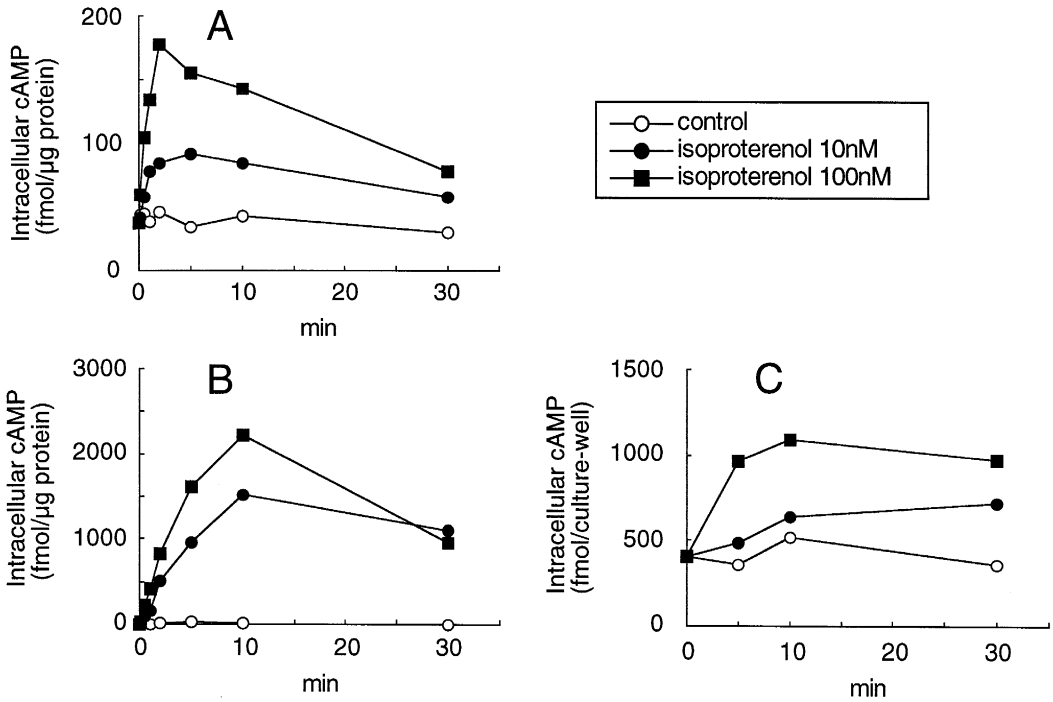

Fig. 1. Effects of isoproterenol on the intracellular cAMP levels in cultured rat cortical neurons (A), rat astrocytes (B) and human vascular smooth muscle cells (C). Cells in the control groups were incubated in the balanced salt solution. Data are presented as the means of 2–3 samples.

isoproterenol increased and reached a plateau at 10 min, whereas the cAMP level continued to increase for at least 30 min in response to 10 nM isoproterenol (Fig. 1C).

3.2. Effects of selective phosphodiesterase inhibitors on the intracellular cAMP levels in cultured cortical neurons in the absence or the presence of isoproterenol

The effects of selective phosphodiesterase inhibitors on the intracellular cAMP level in cortical neurons were investigated in the absence or presence of isoproterenol (Fig. 2). None of the inhibitors showed any effect on the intracellular cAMP level in the cortical neurons in the absence of isoproterenol (Fig. 2A). However, rolipram, Ro 20-1724 and EHNA significantly enhanced the isoproterenol-induced increase in cAMP level ($P < 0.05$) (Fig. 2B). Vinpocetine, milrinone and zaprinast had no effects on the intracellular cAMP level under stimulated as well as resting conditions. Under conditions in which the cells were incubated for 5 min with each phosphodiesterase inhibitor and 10 nM isoproterenol, the result was similar except that the effect of EHNA was not significant (data not shown).

3.3. Effects of selective phosphodiesterase inhibitors on the intracellular cGMP levels in cultured cortical neurons in the absence or the presence of guanylyl cyclase activator

The effects of selective phosphodiesterase inhibitors on the intracellular cGMP level in cortical neurons were

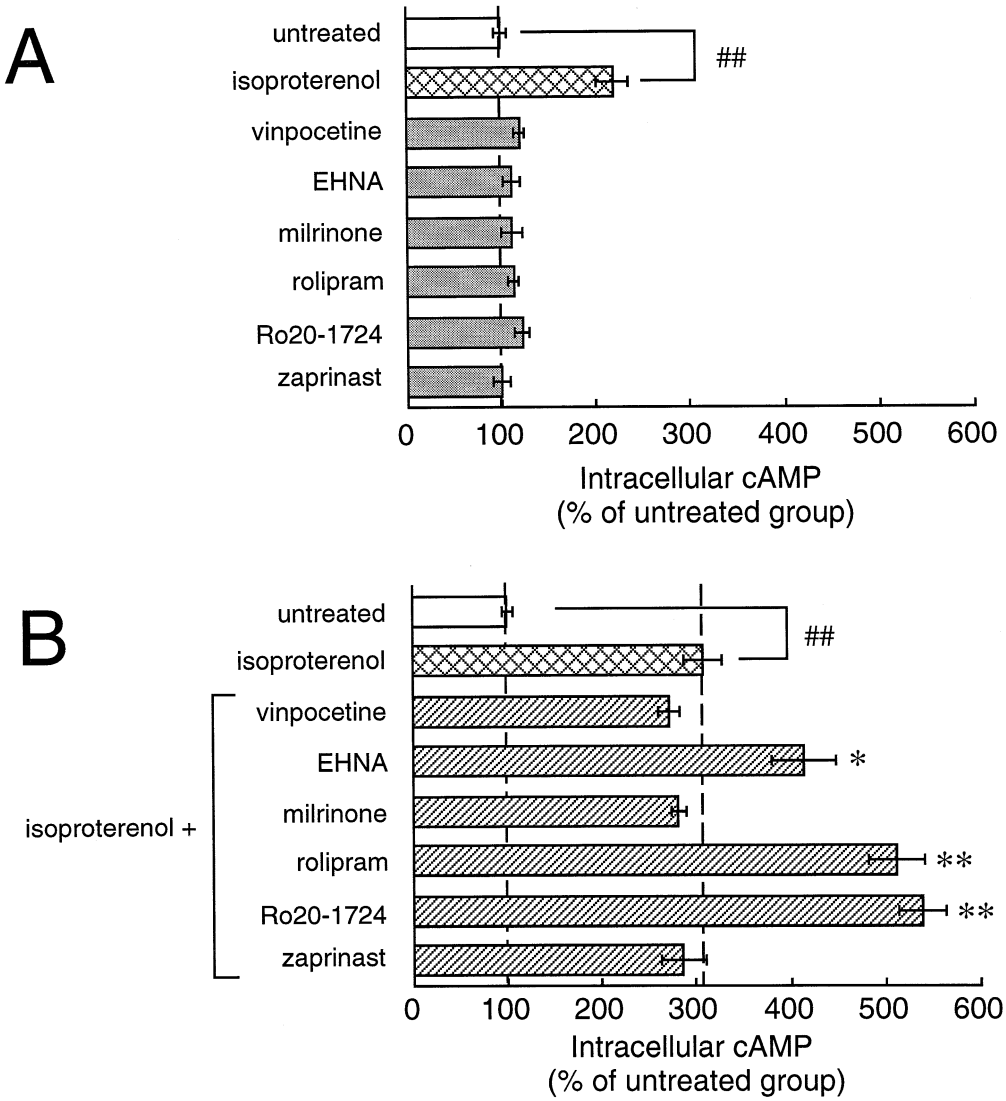

Fig. 2. Effects of selective phosphodiesterase inhibitors on the intracellular cAMP levels in cultured cortical neurons in the absence (A) or the presence (B) of isoproterenol. Cells were incubated for 30 min in the balanced salt solution containing 60 μM vinpocetine, 15 μM EHNA, 1 μM milrinone, 1 μM rolipram, 10 μM Ro 20-1724 or 1.5 μM zaprinast, respectively, with or without isoproterenol. The concentrations of isoproterenol tested were 10 nM in (A) and 100 nM in (B). The cAMP levels were expressed as percentages of the value in the untreated group in each experiment. Data are presented as the means ± S.E.M. (A, $n = 6$; B, $n = 7$). Symbols are as follows: $^{\#\#}$ $P < 0.01$ versus the untreated group in each graph, * $P < 0.05$, ** $P < 0.01$ versus the control group treated with isoproterenol alone in (B).

investigated in the absence or presence of stimulation with guanylyl cyclase activator (Fig. 3). EHNA significantly increased the intracellular cGMP level in the unstimulated neurons ($P < 0.01$, Fig. 3A) and in the neurons stimulated with NONOate ($P < 0.01$, Fig. 3B). Rolipram, Ro 20-1724, milrinone and zaprinast had no effect on the cGMP level. Vinpocetine decreased the intracellular cGMP level under unstimulated conditions ($P < 0.01$, Fig. 3A).

3.4. Effects of rolipram on the intracellular cAMP level in cultured cortical neurons, astrocytes and vascular smooth muscle cells in the presence of isoproterenol

The effect of rolipram on intracellular cAMP in cortical neurons, astrocytes and vascular smooth muscle cells were investigated for a wide range of concentrations (1, 10, 100 and 1000 nM). In the absence of isoproterenol, the intracellular cAMP level in the astrocytes was undetectable and rolipram had no effect on the intracellular cAMP levels in any of the cells (data not shown). Rolipram enhanced the increase of the intracellular cAMP levels in all of cells in the presence of isoproterenol (Fig. 4). The minimum effective concentrations of rolipram were 10 nM for the cortical neurons ($P < 0.05$, Fig. 4A), 100 nM for the astrocytes ($P < 0.05$, Fig. 4B) and 1000 nM for the vascular smooth muscle cells ($P < 0.05$, Fig. 4C), respectively, in the presence of 100 nM isoproterenol. Rolipram at 10 nM or more also significantly enhanced the effect of 10 nM isoproterenol on the intracellular cAMP level in the corti-

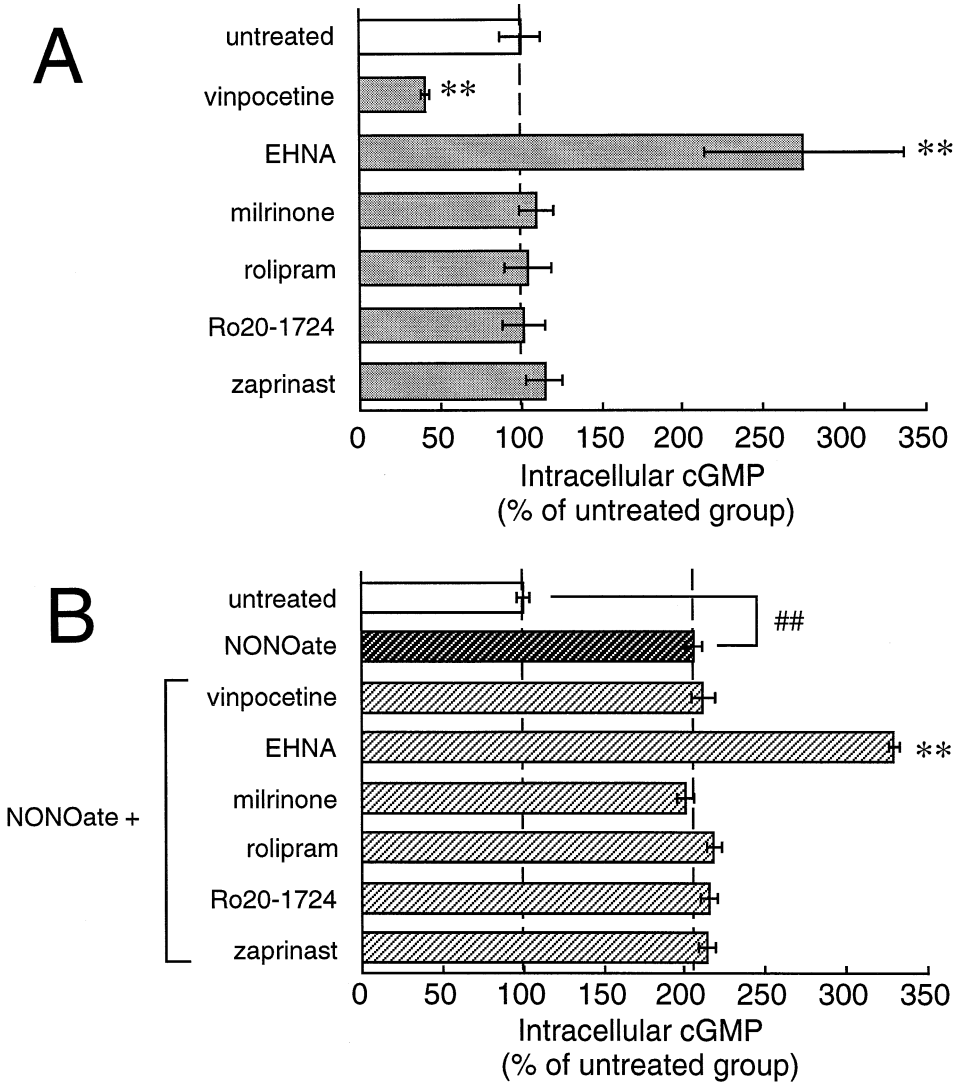

Fig. 3. Effects of selective phosphodiesterase inhibitors on the intracellular cGMP levels in the cortical neurons in the absence (A) or the presence (B) of a guanylyl cyclase activator. Cells were incubated for 30 min in the balanced salt solution containing each phosphodiesterase inhibitor at the same concentrations as in Fig. 1 with or without 100 nM NONOate, a nitric oxide donor. The cGMP levels were expressed as percentages of the value in the untreated group in each experiment. Data are presented as the means ± S.E.M. (A, $n = 6$; B, $n = 7$). Symbols are as follows: $^{\#\#}$ $P < 0.01$ versus the untreated group in each graph, ** $P < 0.01$ versus the control group treated with NONOate alone in (B).

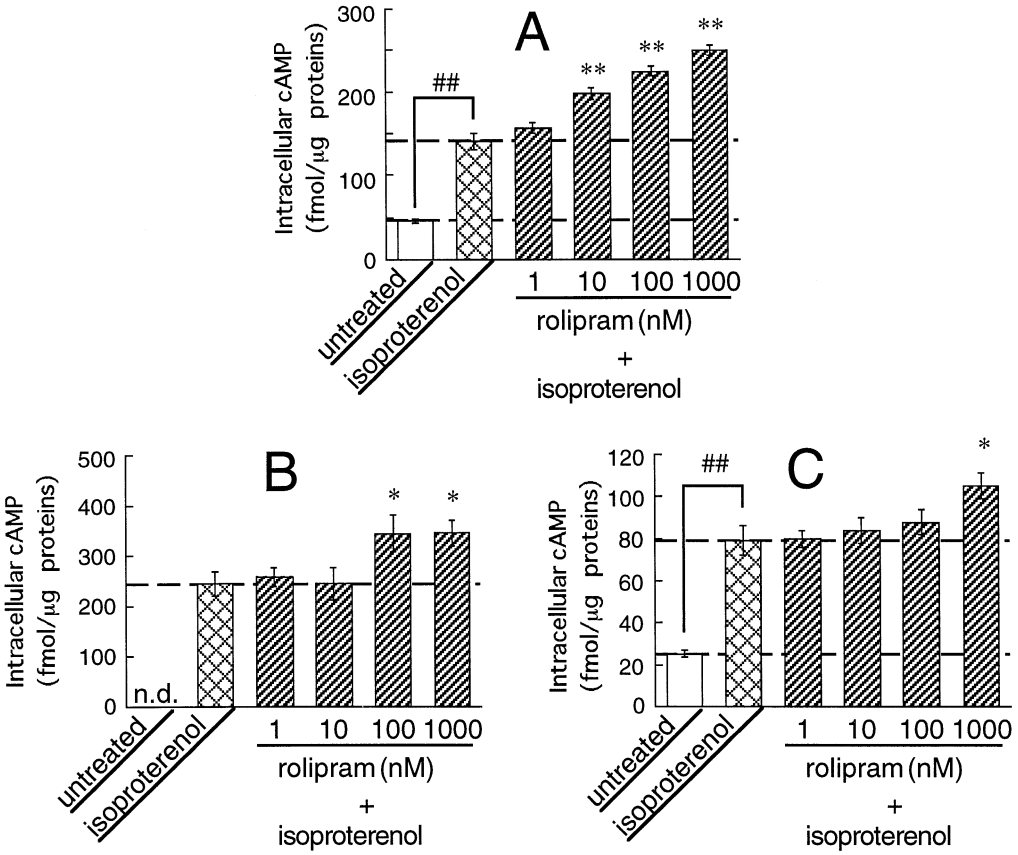

Fig. 4. Effects of rolipram on the intracellular cAMP level in cortical neurons (A), astrocytes (B) and vascular smooth muscle cells (C) in the presence of 100 nM isoproterenol. Cells were incubated for 30 min. Data are presented as the means ± S.E.M. ($n = 9$). Symbols are as follows in each graph: $^{\#\#}$ $P < 0.01$ versus the untreated group, * $P < 0.05$ and ** $P < 0.01$ versus the control group treated with isoproterenol alone. n.d.: not detected.

cal neurons after 5 min incubation ($P < 0.05$, $n = 9$, data not shown).

4. Discussion

In the present study, the selective inhibitors of phosphodiesterase type 4 and type 2 enhanced the β-adrenoceptor-mediated increase of intracellular cAMP in cultured cortical neurons. This finding indicates that phosphodiesterase type 4 and type 2 are major phosphodiesterase isozymes which catabolize cAMP elevated by β-adrenoceptor activation in the cortical neurons. A phosphodiesterase type 2 inhibitor, EHNA, increased intracellular cGMP levels in the neurons with or without stimulation of guanylyl cyclase whereas the phosphodiesterase type 4 inhibitors, rolipram and Ro 20-1724, showed no effect on the cGMP levels. Thus, inhibition of phosphodiesterase type 4 appeared to be an effective strategy to selectively raise the cAMP levels in the cortical neurons. Phosphodiesterase type 4 inhibitors did not affect the basal cAMP level in the absence of isoproterenol whereas they enhanced the cAMP increase induced by stimulation with isoproterenol. Therefore, phosphodiesterase type 4 inhibitors seem to be effective only when the neurons receive cAMP elevating signals. Thus, phosphodiesterase type 4 inhibitors are expected to amplify the cAMP-mediated signals in functioning neuronal networks.

Reported K_i or IC_{50} values of rolipram for recombinant phosphodiesterase type 4 isozymes are in the range of 0.06 to 2.2 μM (Müller et al., 1996). These facts suggest that different kinds of cells which express these isozymes may show varied reactivity to rolipram. Recently, several biological functions were demonstrated to be modulated by low concentrations of rolipram, namely, enhancement of acid secretion in rabbit gastric glands ($EC_{50} = 13$ nM) (Barnette et al., 1995) and inhibition of tumor necrosis factor-α release from lipopolysaccaride-stimulated macrophages ($EC_{50} = 12$ nM) (Genein et al., 1995). These functions are supposed to be mediated by the cAMP enhancement resulting from phosphodiesterase type 4 activity with high sensitivity to rolipram. In the present study, rolipram induced the cAMP enhancement in β-adrenoceptor-stimulated neurons at a concentration of 10 nM or more, which is direct evidence for the existence of rolipram high-sensitive phosphodiesterase type 4 in cortical neurons. We also found that rolipram enhances dopamine biosynthesis and survival of forskolin-treated

primary-cultured mesencephalic dopaminergic neurons over the same concentration range (unpublished data). Thus, rolipram high sensitive phosphodiesterase type 4 may also exist in neurons other than those of the cerebral cortex, playing a major role in regulating cAMP turn-over in the central nervous system.

The isoproterenol-induced increase of the intracellular cAMP levels in astrocytes and vascular smooth muscle cells were also enhanced by rolipram, but the minimum effective concentrations of rolipram were ten times or more higher than that in the cortical neurons. These findings suggest the diversity of phosphodiesterase type 4 isozymes in a variety of cells. Thus, high sensitivity to rolipram appeared to be a unique character of the phosphodiesterase type 4 isozymes in the cortical neurons. Rolipram enhances the effects of cAMP-elevating drugs on cardiac contractility (Muller et al., 1990) and vascular smooth muscle relaxation (Komas et al., 1991). However, the present findings indicated that rolipram, at lower doses, selectively affects cAMP levels in neurons without substantial effects on the cardiovascular system. This may be useful information with regard to the clinical use of phosphodiesterase type 4 inhibitors in the central nervous system for avoiding severe side effects in the cardiovascular system.

EHNA increased both intracellular cAMP and cGMP levels. Since EHNA is known as an inhibitor of adenosine deaminase (Muraoka et al., 1990), it may also increase adenosine which is generated from released cAMP (Rosenberg et al., 1994) or other sources and cause both increase and decrease of intracellular cAMP via stimulation of adenosine A_2 and A_1 receptors, respectively. On the other hand, the effect of EHNA on the intracellular cGMP levels in the present study suggests that, among phosphodiesterases, phosphodiesterase type 2 plays a predominant role in regulating the intracellular cGMP turn-over in cortical neurons under both resting and stimulated conditions. Since phosphodiesterase type 2 is known to be a cGMP-stimulated phosphodiesterase (Beavo, 1995), it is possible that phosphodiesterase type 2 is activated by cGMP and in turn regulates its homeostatic intracellular levels in cortical neurons. EHNA at the concentration we now used could be regarded to completely inhibit phosphodiesterase type 2 which regulates the homeostatic cGMP turn-over in the absence of an extrinsic guanylyl cyclase activator, and thus it is reasonable that the cGMP elevating effect of NONOate was not synergistic with, but additive to the effect of EHNA. On the contrary, phosphodiesterase type 3, a cGMP-inhibited phosphodiesterase, may be inhibited by spontaneously produced cGMP even in resting neurons. This hypothesis was supported by the experimental findings that the phosphodiesterase type 3 inhibitor, milrinone, showed no effect on either cAMP or cGMP levels in the cortical neurons.

The present findings showed that a phosphodiesterase type 1 inhibitor, vinpocetine, had no effect on intracellular cAMP levels. Since phosphodiesterase type 1 has been known to exist in postsynaptic sites in brain (Ludvig et al., 1991), it seems to play an important role locally in this area. In this context, phosphodiesterase type 1 inhibition may not affect the total amount of cAMP in the cortical neurons.

In conclusion, we found that phosphodiesterase type 4, which shows high sensitivity to rolipram, plays a major role in regulating cAMP turn-over in cortical neurons. Thus, rolipram at low concentrations is expected to cause a selective increase of intracellular cAMP in cortical neurons and to regulate the neuronal functions of the cerebral cortex by enhancing the cAMP-dependent protein kinase cascade.

Acknowledgements

We thank Dr. Kazuho Abe (University of Tokyo) for valuable discussions.

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