Rolipram, a Phosphodiesterase-4-Selective Inhibitor, Promotes the Survival of Cultured Rat Dopaminergic Neurons

Nobuyuki Yamashita, Akiko Hayashi, Jun Baba and Aiko Sawa*

Drug Discovery, Pharmaceutical Research Center, Meiji Seka Kaisha, Ltd., 760 Morooka-cho, Kohoku-ku, Yokohama 222, Japan

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ABSTRACT—We evaluated the effects of rolipram, a selective inhibitor of phosphodiesterase (PDE) 4, on the survival of dopaminergic neurons in 13-day culture. Rolipram did not affect the survival of dopaminergic neurons in the absence of forskolin, but significantly enhanced the survival of dopaminergic neurons in the presence of $10^{-5}$ M forskolin in a concentration-dependent manner ($10^{-8}$–$10^{-5}$ M). Rolipram also enhanced the neurotrophic effect of forskolin on total neurons including dopaminergic and non-dopaminergic neurons at a high concentration ($10^{-5}$ M), but did not affect the survival of cells containing glutamate or $\gamma$-aminobutylic acid. A non-selective PDE inhibitor, 1-isobutyl-3-methylxanthine, caused a marked increase of dopaminergic neurons, whereas selective inhibitors of PDE2 and PDE3 showed far weaker effects. A PDE1 inhibitor, on the other hand, caused non-specific cell death in the presence or absence of forskolin. These findings suggest that rolipram has a potential to enhance the survival of dopaminergic neurons selectively by way of PDE4 inhibition.

Keywords: Rolipram, Phosphodiesterase 4, Forskolin, Dopaminergic neuron, Survival

Neurotrophic factors should be useful in treating neurodegenerative disorders including Alzheimer’s, Huntington’s and Parkinson’s diseases. However, neurotrophic factors do not easily cross the blood-brain barrier (BBB) because most of them are macromolecular proteins (1). Thus, micromolecules that can cross the BBB and produce neurotrophic effects will be extremely effective for therapy of neurodegenerative disorders. Elevated levels of intracellular cAMP appear to promote the differentiation and survival of dopaminergic neurons (2) and protects dopaminergic neurons against the neurotoxic effects of MPP+, a specific dopaminergic neurotoxin (3). Intracellular cAMP increases in response to the activation of adenylyl cyclase and is degraded by cyclic nucleotide phosphodiesterase (PDE), which catalyzes the hydrolysis of cAMP to 5'-AMP (4). Among the PDEs (PDE1–7), PDE1, PDE2, PDE3 and PDE4 are found in the brain (4, 5). We have found that PDE4 plays a predominant role in the regulation of intracellular cAMP levels in cultured cortical neurons, showing high sensitivity for rolipram, a selective PDE4 inhibitor (6). Rolipram crosses the BBB (7) and ameliorates cerebral-ischemia-induced impairments of learning and memory in rats by systemic administration (8). In the present study, we evaluated the effects of rolipram on the survival of dopaminergic neurons using primary cultured mesencephalic neurons.

MATERIALS AND METHODS

Materials

Animals were purchased from Japan SLC (Shizuoka). Rolipram was obtained from Schering AG (Berlin, Germany). Anti-tyrosine hydroxylase (TH) polyclonal antibody, anti-microtuble associated protein 2 (MAP2) monoclonal antibody, vinpocetine and 1-isobutyl-3-methylxanthine (IBMX) were purchased from Chemicon International, Inc. (Temecula, CA, USA), Amersham International plc (Buckinghamshire, UK), Calbiochem-Novabiochem Co. (La Jolla, CA, USA) and Nacalai Tesque, Inc. (Kyoto), respectively. Fetal bovine serum (FBS) and L-15 medium were purchased from Life Technologies, Inc. (Rockville, MD, USA). Dubecco’s modified Eagle medium (DME) and phosphate-buffered saline (PBS) were purchased from Nissui Pharmaceutical Co. (Tokyo). Other drugs were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

* To whom correspondence should be addressed.
**Culture of mesencephalic neurons**

The 15-day-old embryos of Wistar rats were obtained from pregnant rats anesthetized by ether; the mesencephalic tissues of the embryos were dissected in L-15 medium and then incubated in PBS containing 0.25% trypsin and 0.5% glucose for 30 min at 37°C. The mesencephalic cells were dissociated by gentle pipetting in DME containing 1% glucose and 10% FBS, and seeded onto poly-L-lysine-coated, 96-well culture plates (Sumitomo Bakelite Co., Tokyo) or on an astrocyte monolayer prepared as described previously (9) at a plating density of $2 \times 10^5$ cells/cm². The cells were incubated at 37°C in a humidified 5% CO₂-95% air atmosphere.

After 24 hr in culture, the medium was changed to modified DME for serum-free culture (DME containing 1% glucose, 1 mg/ml bovine serum albumin, 5 µg/ml insulin, 5 µg/ml transferrin, 100 µM progesterone and 30 nM Na₂SeO₃), containing rolipram ($10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$ and $10^{-5}$ M) and/or forskolin (an adenylyl cyclase activator, $10^{-3}$ M); and then the cells were cultured for 12 days. Similarly, the following PDE inhibitors were added to culture medium in place of rolipram: vinpocetine (a PDE1 inhibitor, $6 \times 10^{-5}$ M), erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA, a PDE2 inhibitor, $1.5 \times 10^{-5}$ M), milrinone (a PDE3 inhibitor, $10^{-3}$ M) and IBMX (a non-selective PDE inhibitor, $2 \times 10^{-3}$ M). These PDE inhibitors were used at the sufficient concentrations for inhibition of each type of PDE. Medium was changed every 6 days. In some experiments, the cells were cultured for 7 days in the presence of vinpocetine at the concentration of $10^{-6}$, $3 \times 10^{-6}$, $10^{-5}$ or $3 \times 10^{-5}$ M.

**Immunostaining**

Cultured cells were identified by immunostaining. The cells were fixed with 4% paraformaldehyde and stained with anti-TH antibody, anti-MAP2 antibody, a polyclonal antibody for glutamic acid (Glu) or a polyclonal antibody for γ-aminobutylic acid (GABA) by the conventional ABC method (VECTASTAIN ABC kit; Vector, Burlingame, CA, USA). TH-positive cells and MAP2-positive cells were identified specifically as dopaminergic neurons and all neurons, respectively. Glu-positive cells and GABA-positive cells were identified as the neuron containing Glu and GABA, respectively.

**Statistical analyses**

The untreated groups and the positive control groups that were exposed to forskolin alone were compared by Student's or Aspin-Welch's t-test depending on the results of the F-test. Multiple comparison between the
control group and the groups treated with rolipram or the other PDE inhibitors were performed by Dunnett's or Steel's procedure depending on the result of Bartlett's test for the homogeneity of variance. In the experiments under the adenylyl-cyclase-inactivated condition, the control group was an untreated group, while in the experiments under the adenylyl-cyclase-activated condition, the control group was the group treated with forskolin alone.

RESULTS

The number of tyrosine hydroxylase-positive cells (TH-positive cells) in the cultured mesencephalic cells were maximum at 24 hr in vitro and then gradually decreased during the 13 days of culture (Fig. 1). Forskolin significantly increased the number of the TH-positive cells at concentrations of $10^{-5}$ M or more ($n=5$, $P<0.05$, data not shown). Rolipram did not affect the survival of the TH-positive neurons with or without an astrocyte-monolayer in the absence of forskolin (Fig. 2: A and B), but significantly enhanced the survival of the TH-positive cells in the presence of $10^{-7}$ M forskolin in a concentration-dependent manner in the range of $10^{-8} - 10^{-5}$ M (Fig. 3A). The number of the TH-positive cells in the presence of $10^{-5}$ M rolipram was 192% that in the control group, which was treated with $10^{-5}$ M forskolin alone. The minimum effective concentration of rolipram was $10^{-8}$ M ($P<0.01$, Fig. 3A). Forskolin also increased the number of the MAP2-positive cells at a concentration of $10^{-5}$ M ($n=5$, $P<0.05$). Rolipram enhanced the neurotrophic effect of forskolin on the MAP2-positive cells at $10^{-5}$ M (118% that of the control group incubated with $10^{-5}$ M forskolin alone) ($P<0.05$, Fig. 3B). Forskolin with or without rolipram did not affect the number of Glu- or GABA-positive cells (Fig. 3: C and D). IBMX ($2 \times 10^{-5}$ M) increased the number of TH-positive cells to

**Fig. 3.** Effects of rolipram on the survival of the mesencephalic neurons in the presence of forskolin. A: The dopaminergic neurons (the TH-positive cells). B: The total mesencephalic neurons (the MAP2-positive cells). C: The Glu-containing neurons (Glu-positive cells). D: The GABA-containing neurons (GABA-positive cells). Cells were cultured on a poly-l-lysine-coated plate. Data in each graph were derived from separate experiments. The data represent means±S.E.M. ($n=8$), and symbols indicate the following in each graph: *$P<0.05$, **$P<0.01$ vs the untreated group; *$P<0.05$, **$P<0.01$ vs the control group exposed to $10^{-5}$ M forskolin alone.
the same degree as $10^{-5}$ M rolipram ($P < 0.01$, Fig. 4). On the other hand, milrinone promoted the neurotrophic effect of forskolin ($P < 0.01$) but was less effective than rolipram, and EHNA did not show a significant effect on the number of TH-positive neurons (Fig. 4). Vinpocetine caused a marked cell death in the presence or absence of forskolin (Figs. 4 and 5). The toxic effects of vinpocetine was non-selective for cultured mesencephalic cells (data not shown).

DISCUSSION

Rolipram selectively promoted the survival of mesencephalic dopaminergic neurons in the presence of an adenylyl cyclase activator. These findings support the results of the previous studies that showed the neurotrophic effect of agents that elevate intracellular cAMP in dopaminergic neurons (2, 3, 10). In the present study, rolipram seemed to elevate intracellular cAMP levels, which then induced differentiation from immature cells to matured dopaminergic neurons, and supported the survival of the differentiated dopaminergic neurons. The elevated intracellular cAMP activates cAMP-dependent protein kinase (PKA) that regulates many cellular functions by phosphorylation (11). The phosphorylation of a transcription factor, cAMP-response element binding protein (CREB), is one of the putative mechanisms leading to the long-term survival of dopaminergic neurons (12). Phosphorylated CREB binds to a cAMP-response element (CRE) on DNA, promoting cAMP dependent transcription of the various kinds of genes and produces such as enzymes, cytoskeleton, receptors, ion-channels and neuromodulators (13). The mechanism of the neurotrophic effects of the elevated intracellular cAMP in dopaminergic neurons remains unclear, but it causes the increase of dopamine-uptake and expression of tyrosine hydroxylase in mesencephalic dopaminergic neurons (2). Intracellular cAMP regulates the expression of neurotrophins including brain-derived neurotrophic factor (BDNF), a typical neurotrophic factor for dopaminergic neurons (14) in the brain (15) and in cultured astrocytes.
Rolipram promotes survival of DA neurons

(16). In the present study, rolipram did not show any neurotrophic effect on the dopaminergic neurons in the absence of an adenylyl cyclase activator, even when the cells were cultured on the astrocyte-monolayers. This finding suggests that intracellular cAMP levels in the dopaminergic neurons and the astrocytes are not elevated by rolipram alone.

Rolipram also increased the number of the MAP2-positive cells, namely, all types of neurons including dopaminergic and non-dopaminergic neurons. However, the number of the MAP2-positive cells in the group exposed to 10^{-5} M rolipram was approximately 120% of the control level, which was substantially less than that of TH-positive cells in the same condition (190% of the control group). Furthermore, the number of neurons containing Glu or GABA were not affected by forskolin with or without rolipram. These findings indicate that rolipram has a potential to enhance the survival of the dopaminergic neurons selectively.

The effects of PDE inhibitors other than rolipram on the number of TH-positive cells suggest that PDE4 plays a major role in regulating intracellular cAMP level with regard to survival of the dopaminergic neurons. Milrinone, a PDE3-selective inhibitor, has been reported to inhibit PDE4 at high concentration (K_i=13 \mu M) (5), thus the effect of milrinone in the present study seems to be partially derived from PDE4 inhibition. The non-specific toxic effect of vinpocetine suggests that PDE1 is associated with apoptosis in neurons, since PDE1 inhibition had reported to induce apoptosis in human leukemia cells (17). However, the non-selective PDE inhibitor IBMX, which also inhibits PDE1, showed a neurotrophic effect on dopaminergic neurons without any toxic effect on neurons under the adenylyl-cyclase-activated condition. It remains to be elucidated whether an extremely high level of intracellular cAMP could rescue the neurons from cell-death associated with PDE1 inhibition.

In conclusion, we showed that rolipram, a selective inhibitor of PDE4, selectively promoted the survival of cultured mesencephalic dopaminergic neurons in the presence of an adenylyl cyclase activator. Rolipram has potential for the treatment of neuronal disorders associated with hypofunction in dopaminergic neurons by way of PDE4 inhibition.

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