Long-range Signaling in Growing Neurons after Local Elevation of Cyclic AMP-dependent Activity

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Abstract. Cyclic AMP-dependent activity at the growth cone or the soma of cultured *Xenopus* spinal neurons was elevated by local extracellular perfusion of the neuron with culture medium containing 8-bromoadenosine 3′,5′-cyclic monophosphate (8-br-cAMP) or forskolin. During local perfusion of one of the growth cones of multipolar neurons with these drugs, the perfused growth cone showed further extension, while the distant, unperfused growth cones were inhibited in their growth. Local perfusion of the growth cone with culture medium or local perfusion with 8-br-cAMP at a cell-free region 100 μm away from the growth cone did not produce any effect on the extension of the growth cone. Reduced extension of all growth cones was observed when the perfusion with 8-br-cAMP was restricted to the soma. The distant inhibitory effect does not depend on the growth of the perfused growth cone since local coperfusion of the growth cone with 8-br-cAMP and colchicine inhibited growth on both perfused and unperfused growth cones, while local perfusion with colchicine alone inhibited only the perfused growth cone. The distant inhibitory effect was abolished when the perfusion of 8-br-cAMP was carried out together with kinase inhibitor H-8, suggesting the involvement of cAMP-dependent protein kinase and/or its downstream factors in the long-range inhibitory signaling. Uniform exposure of the entire neuron to bath-applied 8-br-cAMP, however, led to enhanced growth activity at all growth cones. Thus, local elevation of cAMP-dependent activity produces long-range and opposite effects on distant parts of the neuron, and a cytosolic gradient of second messengers may produce effects distinctly different from those following uniform global elevation of the messenger, leading to differential growth regulation at different regions of the same neuron.

A unique feature of neuronal morphology is the large distance a single neuron may traverse. Various regions of the neuron are likely to be exposed to and activated by different external agents. During development, the growing nerve process encounters localized extracellular guidance signals before it arrives at the target cell (Letourneau, 1985; Landmesser, 1988; Bray and Hollenbeck, 1988). Once synaptic connections are made, various presynaptic nerve terminals of the same neuron may receive different retrograde signals from different postsynaptic target cells. Localized reception of external signals by neuronal surface receptors may lead to a local elevation of second messengers within the cytoplasm, which in turn regulates the differentiation and survival of the neuron (Purves, 1986). Since region-specific activation is likely to be a common feature in the neuron's interactions with the environment, it is of interest to address several questions concerning the response of a neuron to localized signals. For example, does local elevation of second messengers produce only local effects? How is the intracellular spread of the second messengers regulated? Does the neuron react differently to localized signals than to signals that are uniformly received over the entire cell surface?

To begin to address these questions, we have investigated the effect of local elevation of cAMP-dependent activity on neurite growth. Local extracellular perfusion was used to apply a membrane-permeable cAMP analogue or forskolin to one of the growth cones of cultured *Xenopus* spinal neurons, and we examined the effects on the growth of perfused growth cones, as well as of distant, unperfused growth cones. The effects on neurite growth after uniform global elevation of cAMP activity were also examined and compared to those induced by local activation.

Materials and Methods

Culture Preparation

Cultures of embryonic *Xenopus* spinal neurons were prepared by methods previously reported (Spitzer and Lamborghini, 1976; Anderson et al., 1977; Tabti and Poo, 1991). Briefly, the neural tube tissue of 1-d-old *Xeno-*

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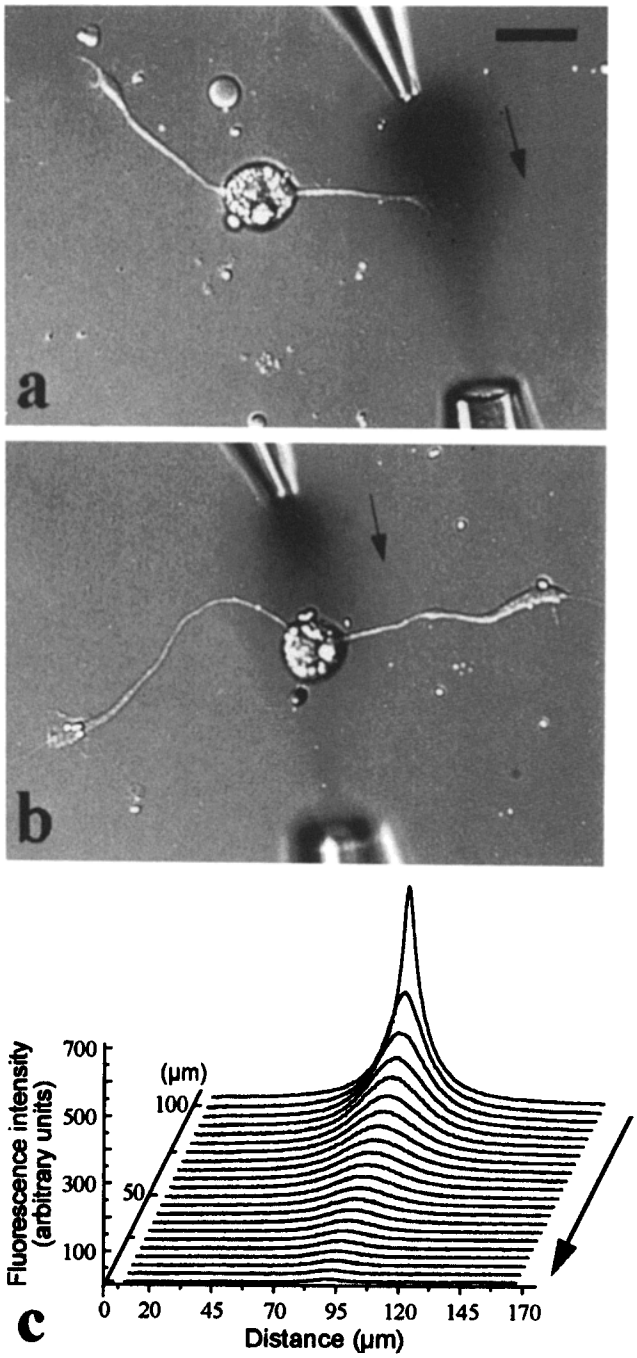


Figure 1. Local perfusion of *Xenopus* spinal neurons at the growth cone (*a*) and at the soma (*b*). Trypan blue was added in the perfusion medium to illustrate the pattern of perfusion flow. The perfusion was introduced by a pair of outflow (*upper*) and suction (*lower*) micropipettes. In (*c*), results from quantitative measurements of the profile of the perfusion flow are shown. Fluorescent dye 5-carboxyfluorescein was used instead of Trypan blue, and the fluorescence intensity measured along equally spaced lines perpendicular to the flow was determined from fluorescence images collected after the onset of the flow. Arrows indicate the direction of the local flow. Bar, 50 μm.

pus embryos (stage 20–22 according to Nieuwkoop and Faber, 1967) was dissociated in Ca^{2+}-Mg^{2+}-free saline supplemented with EDTA (115 mM NaCl, 2.5 mM KCl, 10 mM Hepes, and 0.5 mM EDTA, pH 7.6) for 15–20 min, plated on glass coverslips, and incubated at room temperature (20–22°C) for 6–10 h before the experiments. The culture medium consists of 50% (vol/vol) Leibovitz L-15 medium (Sigma Immunochemicals, St. Louis, MO), 1% fetal calf serum (GIBCO BRL, Gaithersburg, MD) and 49% Ringer's solution (115 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, and 10 mM Hepes, pH 7.6). All experiments were carried out at room temperature.

Local Perfusion

Two glass micropipettes were used to produce a local perfusion flow across a specific region of the neuron. The outflow pipette (opening = 2 μm) and suction pipette (opening = 10 μm) were positioned 100–150 μm apart, and small positive and negative pressures were applied to the two pipettes, respectively, by leveling of two separate water reservoirs connected to the pipettes through fluid-filled tubings. The flow rate of the medium was set at ~20–30 μm/s, as monitored by the flow of cell debris or particles in the culture medium at the center of the perfused area. The pattern of local flow created near the growth cone and at the soma was revealed by using Trypan blue as a marker of the perfusion medium (see Fig. 1, *a* and *b*). Quantitative measurement of the profile of the local flow produced by this perfusion method was carried out by fluorescence imaging of a fluorescent dye, 5-carboxyfluorescein (Fig. 1 *c*). The fluorescence intensity along equally spaced lines perpendicular to the flow was measured from the fluorescence images collected during stable flow. It is clear that the flow is essentially restricted within a region of 50–75 μm in width (widest near the tip of the outflow pipette, narrowest near the tip of the suction pipette). Outside the region, the fluorescence intensity drops to a level below the detection limit. Once the perfusion parameters were set, no dye was used to avoid possible side effects of the dyes.

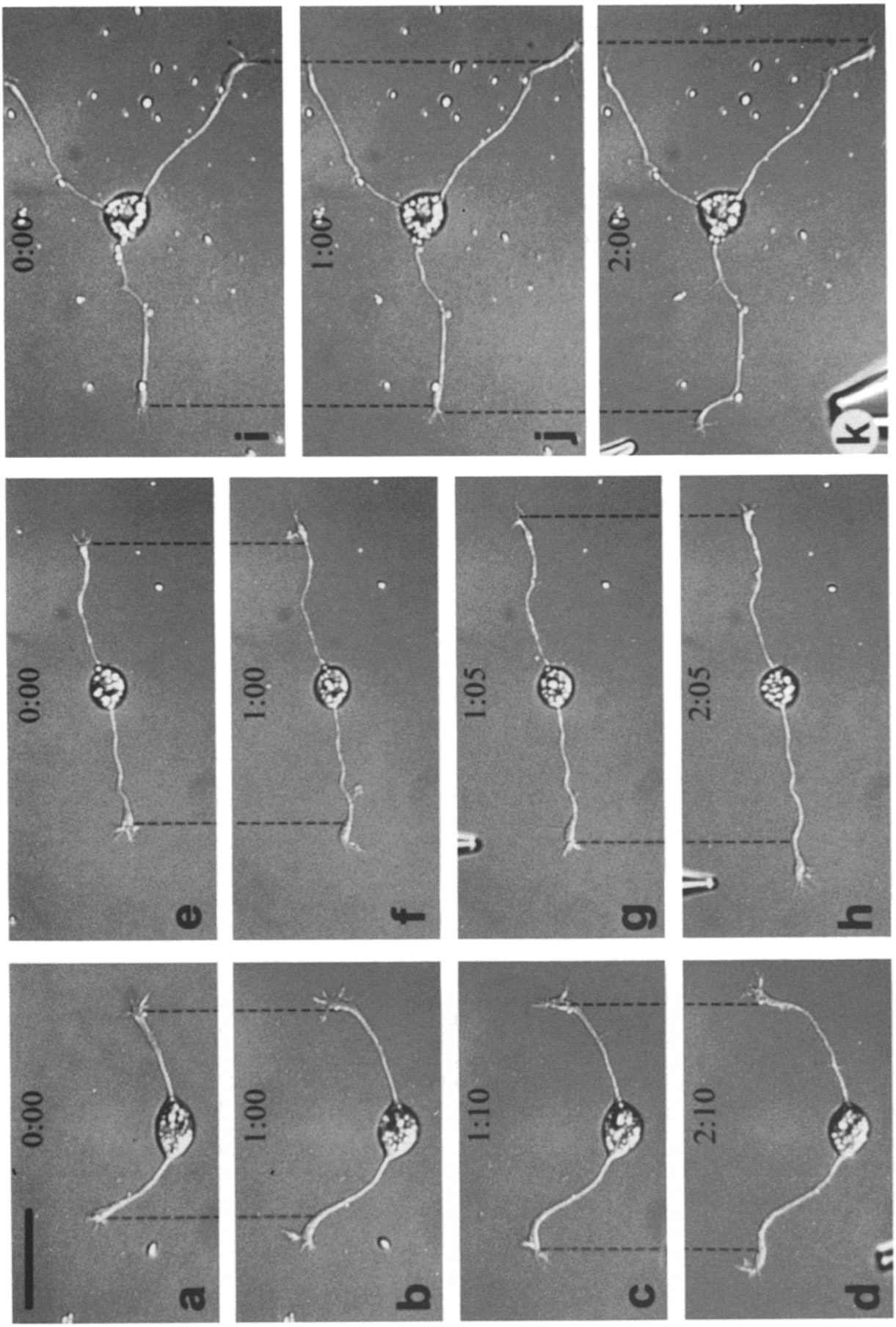
Perfusion medium was made of regular culture medium with or without addition of the following chemicals. 8-bromoadenosine 3′,5′-cyclic monophosphate (8-br-cAMP)[1], a water-soluble, membrane-permeable analogue of cAMP (Muneyama et al., 1971), and colchicine, a microtubule-disrupting agent (Salmon et al., 1984), were purchased from Sigma. Forskolin derivative 7β-deacetyl-7β-[γ-(morpholino)-butyryl]-hydrochloride, which is reported to have improved stability and water solubility over its natural form (Laurenza et al., 1987), and H-8, a potent inhibitor of cAMP-dependent kinases (Higawara et al., 1987), were purchased from RBI (Natick, MA).

Analysis of Neurite Extension

As a standard protocol, extension of all neurites of bi- or multipolar neurons was first monitored for a period of 1 h to determine the control extension rate for each neurite before the perfusion. Local perfusion of the growth cone or soma was then performed for a period of 1 h. Growth cone activity

1. *Abbreviations used in this paper*: 8-br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; CCD, charge-coupled device.

Figure 2. Effects of local perfusion on neurite extension. (*a–d*) Local perfusion of one of the growth cones of a bipolar neuron with culture medium. The neurite extension was first monitored over the 1-h control period in the absence of perfusion (*a*, the onset; *b*, the end). The growth cone on the left was then perfused with culture medium for a period of 1 h (*c*, the onset; *d*, the end). (*e–h*) Similar to *a–d*, except that one of the growth cones was locally perfused with medium containing 0.3 mM 8-br-cAMP. (*e* and *f*) Onset and end of 1-h control period (without perfusion). (*g* and *h*) Onset and end of 1-h perfusion period. (*i–k*) Local perfusion of one of the growth cones of a tripolar neuron with medium containing 0.3 mM 8-br-cAMP. (*i* and *j*) Onset and the end of 1-h control period without perfusion. (*j* and *k*) Onset and the end of 1-h perfusion period. Dashed lines connect corresponding points of the same growth cone at different times during the experiment. Digits represent the time (h:min) after the onset of experiment. Bar, 50 μm.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was monitored by differential interference contrast optics equipped with either a video camera or a cooled charge-coupled device (CCD) camera-based imaging system (STAR I CCD camera; Photometrics Ltd., Tucson, AZ). Images of the neuron at different time points during control and perfusion periods were acquired and saved either on videotape or on hard disk for later analysis and processing. For digital microscopic images acquired with the cooled CCD imaging system, the net extension of neurites during control and perfusion periods was measured directly by calculating the pixel numbers along the neurite trajectory. For the images acquired with the video camera, the net neurite extension during control and perfusion periods was measured from tracings of the video images using a digitizing pad (Hipad; Houston Instruments, Austin, TX). For all measurements, the tip of the growing neurite was defined as the distal leading edge of the "palm" of the growth cone, without considering the filopodial extension. In some experiments, effects of bath application of 8-br-cAMP on neurite extension were examined by tracing the entire trajectory of the neurite, including all its branches, with the digitizing pad.

Results

Local Perfusion of the Growth Cone with 8-br-cAMP

Within hours after cell plating, many cultured *Xenopus* spinal neurons begin active neurite outgrowth. Isolated neurons with two or more neurites in 6–10-h cultures were used for the present study. In the absence of perfusion (Fig. 2, *a*, *b*, *e*, *f*, *i*, and *j*), the neurites extend an average length of 12.6 ± 0.7 μm (SEM, $n = 198$) over the 1-h control period. When the growth cone of one neurite was locally perfused for 1 h with culture medium containing 8-br-cAMP, the perfused growth cone showed enhanced extension, while the other unperfused growth cone became inhibited in its motility (Fig. 2, *g*, *h*, and *k*). This inhibitory effect on the distant unperfused growth cones was observed with bipolar neurons, as well as neurons bearing more than two neurites. As shown in Fig. 2, *i*–k, local perfusion of one growth cone of a tripolar neuron with 8-br-cAMP completely inhibited the extension of the two unperfused growth cones. Perfusion of the growth cone with culture medium alone had no effect on the extension of either perfused or unperfused growth cones (Fig. 2, *c* and *d*).

To better depict the asymmetry in neurite extension after local cAMP perfusion of the growth cone for a large number of neurons, histograms of net neurite extension during the control and perfusion periods of all growth cones are presented in Fig. 3. During the 1-h control period (no perfusion), normal extension was observed for all growth cones (Fig. 3, *a* and *b*), while after the local perfusion, the extension of the perfused growth cones was slightly but significantly elevated (Fig. 3 *c*) compared to that before the perfusion (Fig. 3 *a*), while extension of the unperfused growth cones was largely inhibited (Fig. 3 *d*). Most of the unperfused growth cones stopped extension and some of the unperfused growth cones actually retracted during the 1-h perfusion period (Fig. 3, *d* and *f*, negative values). The data are also summarized in Table I. For 24 neurons examined, the average neurite extension of cAMP-perfused growth cones during the 1-h perfusion period was 17.0 ± 1.6 μm (SEM), which was significantly longer than that during the 1-h control period of the same neurites (12.5 ± 1.8 μm; SEM, $P < 0.01$, two-tail paired *t* test). However, the average neurite extension of unperfused growth cones dropped from 12.8 ± 1.2 μm (SEM) during the 1-h control period to −3.1 ± 1.8 μm (SEM) during the 1-h perfusion period, which was highly significant ($P < 0.001$, two-tail paired *t* test). On the other hand, for 14 neurons examined, local perfusion of the growth cone with culture medium alone did not have any effects on the extension of either the perfused or unperfused growth cones (Fig. 3 *e* and Table I), indicating that the observed effects were not caused by nonspecific mechanical effects of the perfusion. Furthermore, local perfusion with 0.3 mM 8-br-cAMP that was performed at a cell-free region away from the neuron (100 μm away from one of the growth cones) did not produce any effects on the neurite extension of the growth cones (see Table I). This suggests that the spread of the drug to the unperfused region resulting from an imperfect perfusion flow, if it exists at all, was not the cause of the inhibitory effect. This is further supported by our results of local perfusion of the growth cone using lower concentrations of 8-br-cAMP. As shown in Table I, 3 μM and

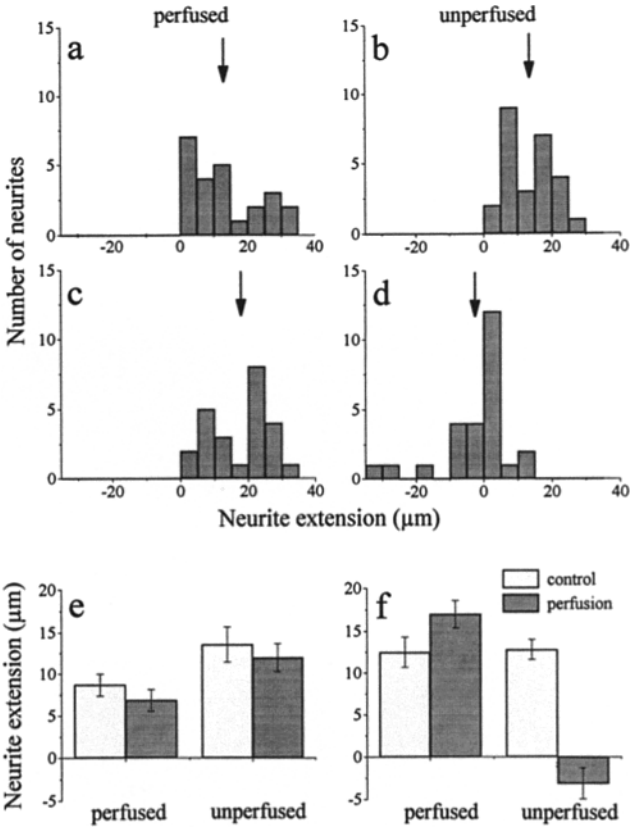


Figure 3. Asymmetric effects on neurite extension induced by local perfusion of the growth cone with 8-br-cAMP. (*a–d*) Distribution of the net length increment of all growth cones from 24 neurons during the 1-h control period (*a* and *b*) and the 1-h perfusion period (*c* and *d*) was plotted in separate histograms. Extension of the perfused growth cone during the 1-h perfusion period (*c*) was increased when compared to that during the control period (*a*), while that of the unperfused growth cone was inhibited (*b*, control period; *d*, perfusion period). Arrows indicate the mean values. (*e*) Average extension (for 14 neurons) after local perfusion of one growth cone with culture medium alone. Average lengths of neurite extension of the perfused and unperfused growth cones during the 1-h control (*open bars*) and 1-h perfusion (*filled bars*) periods were plotted. (*f*) Average extension (for 24 neurons) after local perfusion of one growth cone with 0.3 mM 8-br-cAMP. Note inhibition of unperfused growth cones (*b*, *d*, and *f*). Error bars represent SEM.

*Table I. Effects of Local Perfusion on the Extension of Growth Cones**

Chemicals in perfusion medium	Perfused growth cone			Unperfused growth cone		
	Before perfusion	During perfusion	No. of neurites	Before perfusion	During perfusion	No. of neurites
None (culture medium only)	8.7 ± 1.3	6.9 ± 1.3	14	13.6 ± 2.1	12.0 ± 1.7	15
8-br-cAMP (30 nM)	9.2 ± 1.9	9.4 ± 1.5	12	10.7 ± 2.1	11.8 ± 2.1	14
8-br-cAMP (3 μM)	12.1 ± 1.7	10.2 ± 1.7	11	8.6 ± 1.3	9.3 ± 2.5	12
8-br-cAMP (0.3 mM)	12.5 ± 1.8	17.0 ± 1.6§	24	12.8 ± 1.2	−3.1 ± 1.8§	26
8-br-cAMP (0.3 mM)‡	8.6 ± 0.9	9.8 ± 2.2	12	9.8 ± 2.2	9.3 ± 1.3	14
Forskolin (10 μM)	7.1 ± 1.8	7.9 ± 1.4	9	11.4 ± 1.9	−1.8 ± 3.1§	10
8-br-cAMP (0.3 mM) + H-8 (0.1 mM)	7.5 ± 2.0	7.3 ± 2.1	10	11.9 ± 1.5	7.5 ± 1.5	10
Colchicine (10 μg/ml)	12.2 ± 2.1	0.8 ± 2.7§	12	8.5 ± 1.1	8.3 ± 2.0	14
8-br-cAMP (0.3 mM) + colchicine (10 μg/ml)	13.1 ± 2.1	5.5 ± 3.4§	9	17.9 ± 2.7	4.1 ± 1.6§	9

* Only neurons bearing two or three neurites were used. The extension of all neurites was measured for a period of 1 h before and 1 h during the perfusion. The data represent the net extension of the growth cone during the 1-h period before perfusion and the 1-h period during perfusion (MEAN ± SEM, μm).
‡ Perfusion of 8-br-cAMP was performed 100 μm away from the growth cone at a cell-free area.
§ The extension was significantly different from that was found before the perfusion ($P < 0.05$, two-tail paired t test).

30 nM 8-br-cAMP had no significant effect on the neurite extension of either the perfused or unperfused growth cones.

Local Perfusion of the Growth Cone with Forskolin

In a second series of experiments, the growth cone was perfused with a water-soluble derivative of forskolin instead of 8-br-cAMP. It is known that forskolin can stimulate membrane-bound adenylate cyclase (Laurenza et al., 1987), leading to elevation of the cytosolic cAMP levels. Local perfusion of growth cones with culture medium containing 10 μM forskolin had no significant effect on the rate of neurite extension of the perfused growth cones during a 1-h period ($P > 0.05$, two-tail paired t test, see Fig. 4 and Table I), but it inhibited the neurite extension of distant unperfused growth cones ($P < 0.001$, two-tail paired t test). Fig. 4 *f* summarizes the results obtained from nine bipolar neurons. It is clear that local perfusion of one of the growth cones with forskolin produced significant inhibition on the extension of the unperfused growth cone. During the 1-h perfusion period, most of the unperfused growth cones showed no growth or slight retraction. We also noted that the inhibitory effect produced by local perfusion of the growth cone with 8-br-cAMP or forskolin appeared to be reversible since, in most cases, the unperfused growth cone resumed its motility and extension shortly after the termination of the perfusion (see Fig. 4 *e*).

Local Perfusion of the Soma with 8-br-cAMP

Local perfusion was also applied to the soma of neurite-bearing neurons. As shown in Fig. 5, perfusion of the soma with culture medium containing 0.3 mM 8-br-cAMP resulted in growth inhibition of all existing growth cones. For 20 neurites (from 10 bipolar neurons) examined, the average neurite extension during the 1-h control period was 12.6 ± 1.8 μm and decreased to 4.3 ± 1.2 μm (SEM) during the 1-h period of soma perfusion. This reduction of neurite extension induced by local perfusion of the soma with 8-br-cAMP was statistically significant ($P < 0.01$, two-tail paired t test). Therefore, local elevation of cAMP-dependent activity in the soma produced an inhibitory effect on the extension of all existing growth cones.

Local Perfusion of the Growth Cone with 8-br-cAMP and Colchicine

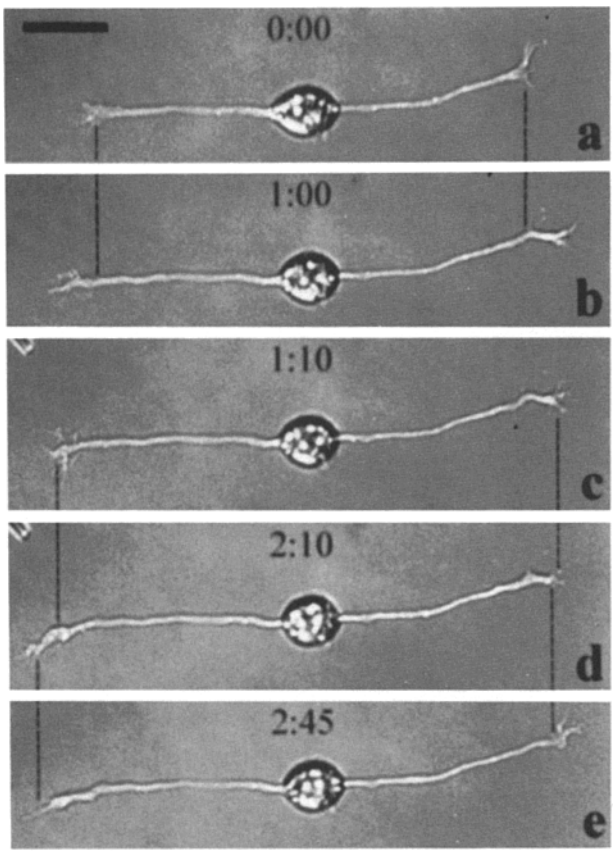
The behavior of distant growth cones may be causally linked to the elevated growth of perfused growth cones. Increasing demands for cellular components (e.g., cytoskeletal proteins) to sustain the elevated growth of the perfused growth cone could reduce the supply for unperfused growth cones, leading to their growth inhibition. This possibility was tested by local perfusion of one of the growth cones of bi- or multipolar neurons with 8-br-cAMP (0.3 mM) together with colchicine (10 μg/ml). As summarized in Table I, local perfusion of a growth cone with colchicine alone inhibited the extension of the perfused growth cone but did not inhibit the distant unperfused growth cone. However, when the growth cone was perfused with 8-br-cAMP together with colchicine, the extension of both perfused and unperfused growth cones was inhibited.

Local Perfusion of the Growth Cone with 8-br-cAMP and H-8

Elevation of the cytosolic cAMP level is known to activate cAMP-dependent protein kinase. Such activation may induce a cascade of cellular events leading to the inhibition of the distant unperfused growth cone. To test the involvement of cAMP-dependent protein kinase in this signaling process, a specific inhibitor of cAMP-dependent protein kinases, H-8, was coperfused with 8-br-cAMP. As shown in Table I, local coperfusion of the growth cone with 8-br-cAMP (0.3 mM) and H-8 (0.1 mM) produced no effect on the neurite extension of the distant unperfused growth cones, as compared to the extension during the control period ($P > 0.05$, paired t test).

Effects of Uniform Exposure to 8-br-cAMP

Our perfusion experiments showed that local elevation of cAMP-dependent activity in a growing neuron produced different effects at local and distant regions of the neuron. For comparison, we have examined the effect of uniform global elevation of cAMP activity on the growth and survival of these *Xenopus* spinal neurons. In the first set of experiments, 8-br-cAMP was added to the culture medium at the



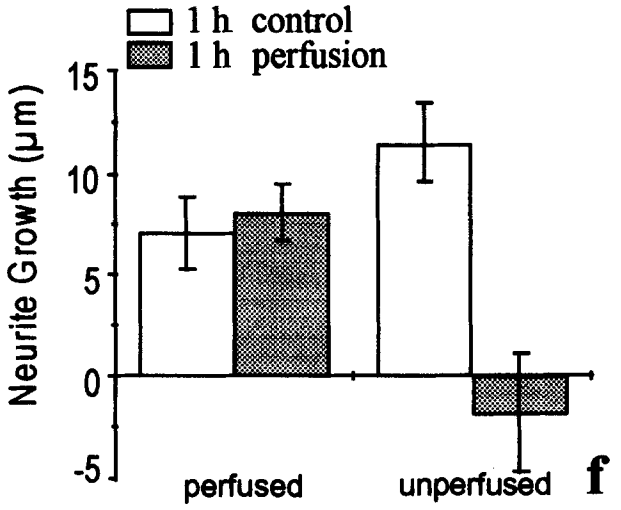
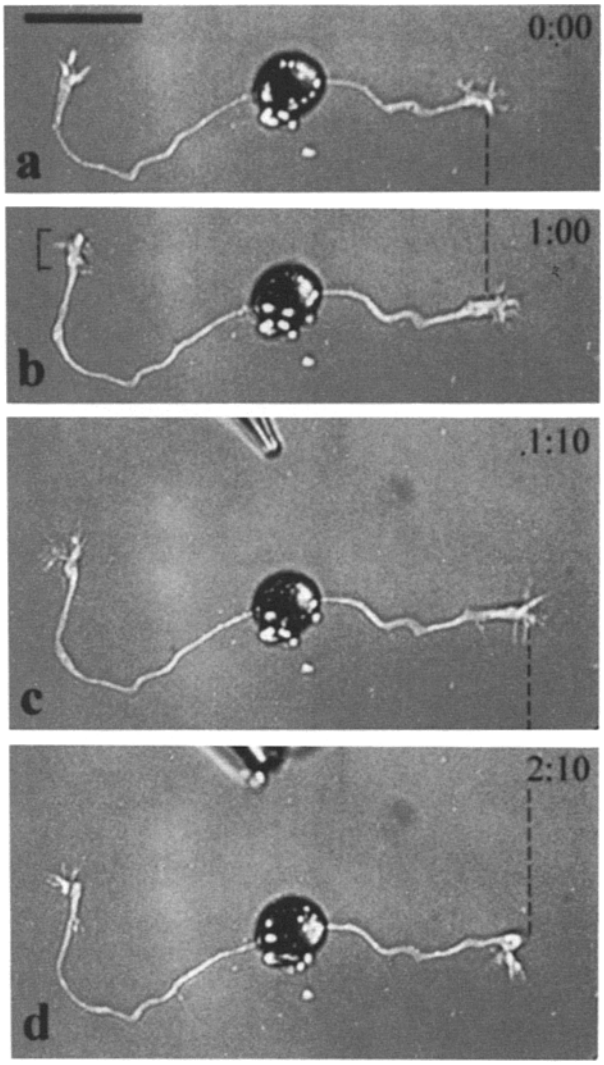


Figure 4. Effects of local perfusion of the growth cone with 10 μM forskolin. (*a* and *b*) A bipolar neuron at the onset (*a*) and the end (*b*) of the 1-h control period. Both growth cones showed active extension. (*c* and *d*) The same neuron at the onset (*c*) and the end (*d*) of the local perfusion of the growth cone on the left with medium containing 10 μM forskolin. The perfused growth cone showed further extension while the unperfused growth cone was inhibited. (*e*) 35 min after the termination of forskolin perfusion, the inhibited growth cone resumed its motile activity. (*f*) Summary of data on the effects of local forskolin perfusion for nine neurons. Average lengths of neurite extension of the same growth cones (perfused and unperfused) *during* the 1-h control (*open bars*) and 1-h perfusion (*filled bars*) periods were plotted. Bar, 50 μm. Error bars represent SEM.



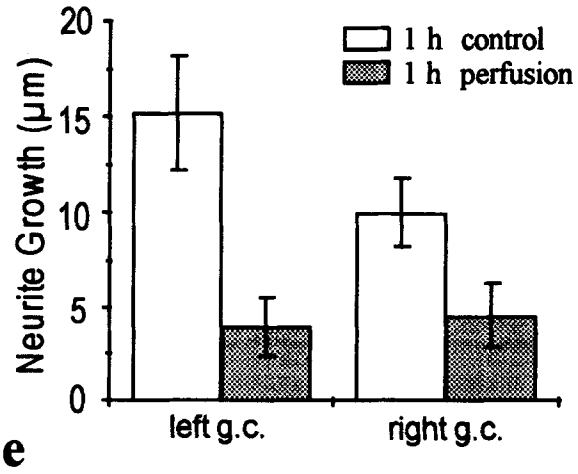


Figure 5. Effects on neurite extension of the growth cone (*g.c.*) by local perfusion of the soma with 8-br-cAMP. All experiments were carried out on bipolar neurons. (*a* and *b*) A bipolar neuron at the onset (*a*) and the end (*b*) of the 1-h control period. The bracket in *b* marks the net extension of the growth cone on the left during the 1-h control period. (*c* and *d*) The same neuron at the onset (*c*) and the end (*d*) of the 1-h local perfusion of the soma with 0.3 mM 8-br-cAMP. The extension of both growth cones was inhibited. (*e*) Average lengths of neurite extension of the same growth cones during the 1-h control (*open bars*) and 1-h perfusion (*filled bars*) periods were plotted for 10 neurons examined. Bar, 50 μm. Error bars represent SEM.

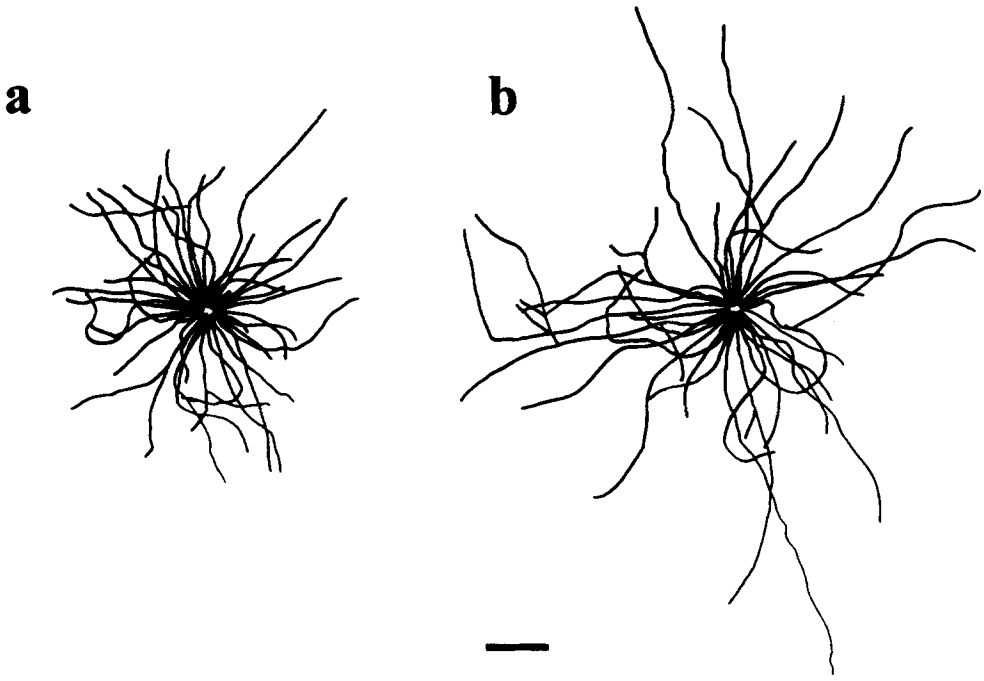


Figure 6. Enhancement of neurite extension by bath-application of 8-br-cAMP. Composite drawings of 30 randomly sampled neurons from 6-h cultures. The center of the soma of each neuron was superimposed at the origin. (*a*) Neurons in a control culture with no 8-br-cAMP added. (*b*) Neurons in a parallel culture supplemented with 0.3 mM 8-br-cAMP. Bar, 20 μm.

time of cell plating. The length of neurite outgrowth in isolated neurons (not in contact with other cells) was measured 6 h after plating the cells. In cultures treated with 0.3 mM 8-br-cAMP, the average neurite length (the entire trajectory of the neurite including all its branches) was 68.4 $\pm$ 3.4 μm (SEM, n = 124, three cultures), which is significantly longer ($P < 0.001$, two-tail t test) than that observed for control untreated cultures (49.6 $\pm$ 2.0 μm; SEM, n = 133, three cultures). Fig. 6 depicts composite tracings from 30 randomly sampled neurons from a 6-h culture supplemented with 8-br-cAMP (Fig. 6 *b*) and from a parallel control culture (Fig. 6 *a*). The effect depended on the concentration of 8-br-cAMP in the medium; no significant effect on the neurite length was observed when the concentration was reduced to 0.01 mM (data not shown).

To determine whether global elevation of cAMP activity could affect growth cone extension after neurite outgrowth had already been initiated, neurite-bearing neurons in 6–10 h cultures were monitored for growth cone extension for 2 h before and after exposure to bath-applied 8-br-cAMP (0.3 mM). We found that the average extension rate of the growth cones was increased from 14.3 $\pm$ 0.9 to 19.2 $\pm$ 1.4 μm/h (SEM, n = 70, $P < 0.005$, two-tail t test) after treatment with 8-br-cAMP. This increase in growth rate apparently reflects growth enhancement of all neurites, and the response was similar to that found in growth cones exposed to local perfusion of 8-br-cAMP. Supplementing the culture with 8-br-cAMP also increased the survival of these *Xenopus* neurons. When neurite-bearing neurons are counted daily during the first 5 d of culture, their number progressively declines. Addition of 8-br-cAMP (0.3 mM) in the culture medium reduced the rate of decline of neurite-bearing neurons (data not shown). Thus our result is consistent with the notion that 8-br-cAMP can promote both neurite extension and neuronal survival in these *Xenopus* cultures.

Discussion

The main finding of this study is that local activation of cAMP-dependent activity produces a long-range inhibitory effect at distant regions of the neuron. Mechanical disruption resulting from the perfusion is unlikely to be the cause of the observed effect since control perfusion using fresh culture medium did not induce any changes in neurite extension at distant growth cones, and coperfusion of 8-br-cAMP with H-8 abolished the effect induced by 8-br-cAMP perfusion alone. The distant inhibitory effect was also unlikely to be caused by a small amount of 8-br-cAMP reached at the distant, unperfused growth cone resulting from an imperfect perfusion flow. Three pieces of evidence argue against the latter possibility. First, quantitative measurements showed that the perfusion flow was strictly localized (see Fig. 1). Second, local perfusion of the growth cones with low concentrations of 8-br-cAMP had no effect on the neurite extension of either the perfused or the unperfused growth cones (see Table I). Third, local perfusion with 8-br-cAMP at a cell-free region away from the growth cones with a distance similar to that between the perfused and unperfused growth cones did not produce any inhibition of neurite extension.

The mechanism responsible for producing the long-range effect is unknown. Since the growth cone perfused with 8-br-cAMP showed increased growth rate, the elevated growth activity may inhibit the extension of the unperfused growth cone, as would be expected if the cellular supply for neurite growth is limited in the neuron (Smalheiser and Crain, 1984). However, local perfusion of the growth cone with forskolin did not increase the growth rate of the perfused growth cone, yet it effectively inhibited the extension of the unperfused growth cone. Previous studies have shown that bath application of forskolin at a concentration of 10–20 μM but not 4 μM increased the growth rate of neurite extension (Lohof

et al., 1992). Because of the rapid decline of the concentration along the perfusion flow according to the fluorescence measurement (see Fig. 1 *c*), the concentration of forskolin focally applied to the growth cone was likely to be much lower than 10 μM, yet it produced inhibitory effect at the distant growth cone. It is possible that the local growth enhancement and distant growth inhibition require different levels of cAMP and that the distant inhibition does not depend on an increased rate of growth of the perfused growth cone. The latter is further supported by the finding of distant inhibition when the extension of the perfused growth cone was blocked by coperfusion of 8-br-cAMP with colchicine. Finally, the growth inhibition of all growth cones was observed when the soma was perfused with 8-br-cAMP. Taken together, we conclude that increased growth rate of the perfused growth cone is not the cause of inhibition at distant growth cones.

Previous studies on growth cones of *Aplysia* bag cell neurons (Forscher et al., 1987) showed that forskolin induces an extension of directed organelle transport into typically organelle-free lamellipodial domains of the growth cone. Elevation of cytosolic cAMP also led to a two- to threefold enhancement of the average rate of organelle transport along the axon (Azhderian et al., 1994). It is possible that local elevation of cAMP level by perfusion increased vesicle transport preferentially towards the cAMP-perfused region, which eventually decreased the cellular activity of the distant, unperfused growth cone and resulted in inhibition. Interestingly, two neurites of an identified *Aplysia* neuron in culture show opposite changes in axonal transport when one of the neurites contacts the appropriate target (Goldberg and Schacher, 1987).

It has been known that elevation of cAMP levels promotes growth cone activity and neurite extension in several culture preparations (Heidemann et al., 1985; Richter-Landsberg and Jastorff, 1986; Rydel and Greene, 1988; Lohof et al., 1992) but inhibits neurite growth in others (Forscher et al., 1987; Mattson et al., 1988). In these previous studies, drugs that elevate cAMP levels were added to the culture medium, which presumably induced uniform global elevation of cAMP activity throughout the neuron. The differences in the effects of global versus local elevation of cAMP activity observed in the present study suggest that two aspects of second messenger signaling in the neuron should be considered: the cytosolic gradient of the second messenger and its basal level throughout the entire neuron. The striking inhibitory effect on distant growth cones produced by local perfusion of one growth cone suggests that a gradient of second messengers, if present, is predominant in determining the cellular response, regardless of whether there is an increased basal level of the messengers. In the absence of a gradient, however, a uniform increase in the basal cAMP activity produced enhanced growth at all neurites in our culture, presumably by a uniform elevation of growth activity throughout the entire neuron. Thus local input to a neuron will result in a qualitatively different effect from that of a global activation. By producing a gradient of cytoplasmic signals, cellular consequences at distant regions may be opposite from that induced by global activation.

What are the cellular events after local elevation of cAMP-dependent activity? How is the activation spread in the neuron? Results of the experiments using H-8 suggest an involvement of protein phosphorylation by protein kinase(s). Based on measurements of the diffusion of soluble proteins within the neurite cytoplasm (Popov and Poo, 1992), the diffusion coefficient of the cAMP-dependent protein kinase (an immediate downstream effector of cAMP) in the cytoplasm is estimated to be $\sim 7 \times 10^{-7}$ cm^2/s. Without degradation or local confinement, the activated proteins should spread to growth cones 200 μm away within 5 min. Thus, to induce differential cellular responses, cellular mechanisms for the confinement and detection of localized cAMP activity are required. At present, the immediate molecular gradient responsible for inducing the asymmetric growth modulation is unknown. In principle, it could be cAMP itself or any one of its downstream effectors, including cAMP-dependent protein kinase and its substrate proteins, e.g., ion channels in the plasma membrane. If effector proteins are involved, local confinement or a stable gradient of the signal could be achieved by the protein's association with immobilized multimolecular complexes, membrane organelles, or the plasma membrane. During natural activation of the neuron by local extracellular ligands, degradation of native second messengers by cytosolic enzymes could help to sharpen the cytosolic gradient, and differential effects at local and distant regions of the neuron may be more pronounced than those observed under the present experimental conditions.

Rapid and local effects of nerve growth factor on the growth cones of cultured neurons have been reported (Seeley and Greene, 1983; Connolly et al., 1987). However, whether and how distant growth cones were affected by local nerve growth factor application was not known. The present results demonstrate that local elevation of cAMP-dependent activity can produce distinctly different effects at local and distant regions of the same neuron. The long-range effect resulting from local neuronal activation has direct implications for neuronal regulation in developing and adult nervous systems. When a growth cone encounters extracellular cues that trigger a local response, distant growth cones of the same neuron may react with an opposite response. In the developing nervous system of the leech, there is evidence for long-ranging signaling between two separate processes of the same neuron; interactions with the target or with another neuron by one neurite lead to changes in the behavior of other neurites (Baptista and Macagno, 1987; Gao and Macagno, 1988). During axon/dendrite differentiation of the hippocampal paramidal neuron in culture, rapid extension of one neurite that is destined to become an axon is accompanied by growth inhibition at other neurites that eventually become dendrites (Goslin and Banker, 1989). After synapse formation, long-range interactions may exist between distant synaptic terminals of the same presynaptic neuron (Clark and Kandel, 1993). Such an interaction may constitute an integral part of the transsynaptic retrograde regulation by the postsynaptic target cell (Harish and Poo, 1992; Dan and Poo, 1994).

In conclusion, the present finding supports the notion that the response of a neuron to a localized signal is global and region specific. Local activation may lead to opposite effects at local and distant regions. This suggests that existence of cytoplasmic mechanisms for the detection and maintenance of a second messenger gradient, as well as for differential responses in accordance with such a gradient. The nature of these mechanisms remains to be elucidated.

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