80. *Functional Synapse Formation between Cultured Neurons of Rat Cerebral Cortex*

Block by a Protein Kinase Inhibitor which does not Permeate the Cell Membrane

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Functions of the brain develop through the formation of synaptic contacts between neurons. Neurons in the brain grow their axons and dendrites and form specific synaptic connections during development. Some synaptic connections are changed functionally and morphologically by environmental stimuli even in adults,¹⁾ and such synaptic plasticity can subserve behavioral changes, including learning and memory. It has also been proposed that the plasticity of synaptic contacts in human association cortex constitutes the cellular mechanism of long-term memory in a "tracing circuit" model.²⁾ Therefore, the molecular mechanisms underlying the formation of synapses are of great interest not only for the developmental biology of brain but also for the neurobiology of learning and memory.

We have developed an experimental system by which the formation of functional synapses between cerebral cortical neurons can be quantitatively observed. Using this assay system, it is now possible to screen biological agents and drugs which inhibit or facilitate synapse formation *in vitro*.⁸⁾ Preliminary results show that long-term application of a protein kinase inhibitor, K-252b, which cannot pass through the cell membrane, blocks synapse formation of cerebral cortical neurons *in vitro*. The data strongly suggest that ecto-protein kinase activity,^{4),5)} i.e. a specific phosphorylation of protein on the outer surface of synaptic membrane, is an essential part of the process of synapse formation.

Materials and methods. *Cell culture:* Cells were dissociated and cultured by a modification of the method of Banker and Cowan.⁶⁾ Cerebral cortices were dissected from 18-day fetal rats. Small pieces of the tissue were incubated at 37°C in Ca²⁺- and Mg²⁺-free phosphate-buffered saline containing 0.02% papain, 0.02% bovine serum albumin, 0.02% D-cysteine HCl and 0.5% glucose for 15 min. The enzyme digestion was repeated once. After separating from the enzyme solution by sedimentation, the sediment was resuspended in a medium consisting of 5% newborn calf serum (Nakashibetsu Serum Center), 5% heat-inactivated horse serum (Gibco) and 90% Dulbecco's modified Eagle's medium (DMEM, Gibco) which contained 1 mM sodium pyruvate, 50 units/ml penicillin G, 0.025 mg/ml streptomycin sulfate and 1.2 mg/ml NaHCO₃. Cells were then mechanically dissociated by trituration through a plastic tipped Pasteur pipette and filtered through a sterile lens paper filter. This suspension was centrifuged at 1,000 r.p.m.

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for 5 min, and the resulting cell pellet was washed and resuspended in the same medium. The final cell suspension (0.5 ml) was plated on poly-L-lysine coated coverslips at a density of 2×10^6 cells/well. Cultures were maintained in a 7% CO_2 at 37°C . The medium was changed every 2–3 days.

Optical monitoring of excitatory synaptic activity: The fluorescence intensities from cultured neurons loaded with a Ca^{2+} indicator, fura-2, was recorded using video-assisted, multi-site fluorometry equipment,¹²⁾ by a previously described method.³⁾

Reagent: Protein kinase inhibitor K-252b was isolated from the culture broth of *Nocardiopsis* sp. K-290.⁷⁾ The agent was dissolved in a small volume of dimethyl sulfoxide (DMSO) and diluted by the incubation solution.

Results and discussion. *Synaptic activity of the cultured neurons:* Cultured neurons prepared from rat cerebral cortex developed spontaneous oscillation of fluorescence intensity corresponding to excitatory synaptic activity, similar to

Fig. 1. Occurrence and maintenance of synaptic activity in cultured rat cerebral cortical neurons. Changes of fluorescence intensity (340 nm) which corresponds to the synaptic activity (see text) are observed in randomly chosen 3 neurons simultaneously by video-assisted, multi-site Ca^{2+} fluorometry at various stages of culture. The numbers show days in culture.

that observed in cultured rat hippocampal neurons.³⁾ As shown in Fig. 1, the neurons start to express some changes in intracellular Ca^{2+} after 3 days in culture. After 5 days in culture, changes of fluorescence intensity become significant. This type of spike-like change of intracellular Ca^{2+} has been shown to be caused by electrical excitation of neurons. Oscillations of fluorescence intensity were observed simultaneously in most of the neurons. The frequency of spontaneous oscillation was gradually increased during further incubation, and after 3 weeks in culture, several peaks of the intensity change pattern coalesced to form plateau-like changes in fluorescence.

As already established in hippocampal neuron culture,³⁾ this oscillation in

the cerebral cortex culture indicates the formation of functional synaptic contacts, since application of tetrodotoxin or of a glutamate receptor antagonist, 2-aminophosphonovaleric acid (APV), reversibly inhibited the increase of fluorescence intensity (data not shown). In hippocampal cultures, lowering of extracellular Ca^{2+} concentration also stopped most of the excitation. However, under the low Ca^{2+} condition, a small number of neurons still show spike-like changes in the fluorescence intensity but independently of each other without oscillation, indicating that they are spontaneously firing neurons.⁸⁾ Spontaneous excitation of pace-maker neurons in culture is transmitted to other neurons through functional synapses, so that all the neurons in the synaptic network excite almost simultaneously. Therefore, it is quite reasonable to use synchroni-

Fig. 2. Effect of a protein kinase inhibitor, K-252b on the synaptic activity of cultured cerebral cortical neurons. Changes of fluorescence intensity (340 nm) were observed in a set of sister cultures 6 days in culture. A: Control, no addition. B: Addition of 50 nM K-252b. C: Addition of 200 nM K-252b, continuously for the whole culture period.

zation of the oscillations in fluorescence intensity as an indication of synapse formation, not only in the hippocampal neuron culture, but also in this cerebral cortical neuron culture. In preliminary experiments using hippocampal neuron cultures, long-term application of several monoclonal antibodies against PC-12 cell surface antigens¹³⁾ abolished synchronization of the oscillations in fluorescence intensity, suggesting that the monoclonal antibodies inhibited the formation of functional synapses.⁸⁾

Application of a protein kinase inhibitor: When 200 nM of protein kinase inhibitor, K-252b, was added to the medium from the beginning of the culture, in which neurons were dissociated, the oscillations in fluorescence intensity were not observed after 6–8 days in the culture (Fig. 2C). In control sister cultures, with or without comparable amount of DMSO, significant oscillations were commonly observed after the same culture period (Fig. 2A). The frequency of

oscillation was lowered when 50 nM of K-252b was applied continuously (Fig. 2B). Since a small number of neurons showed spike-like increases of fluorescence intensity in the culture treated with 200 nM K-252b, the inhibitor does not seem to inhibit the spontaneous activity of pace-maker neurons (data not shown). The data strongly suggest that the protein kinase inhibitor blocked synapse formation between cerebral cortical neurons.

It is interesting to note that, even though the inhibitor, K-252b, can severely block protein kinase activity in *in vitro* assay system [$IC_{50} = 38$ nM],⁷⁾ it generally has no effects on living cells, on which other similar types of inhibitor such as K-252a have significant physiological effects through blocking of protein phosphorylation. More recent studies have indicated that the inhibitor K-252b does not permeate cell membrane (in preparation). This means the site of action of K-252b is the outer surface of the cell membrane.

It has been reported that neuronal membrane has an ecto-protein kinase, i.e. a protein kinase whose substrate site is on the outer surface of the membrane, which phosphorylates N-CAM-like cell adhesion proteins.⁴⁾ The most possible target of the protein kinase inhibitor K-252b is this type of ecto-protein kinase. Another ecto-protein kinase which is regulated by gangliosides has recently been discovered.⁵⁾ The source of substrate for these enzymes is ATP released in the extracellular space. It has already been shown that ATP and adenine nucleotides are released upon stimulation from presynaptic terminals, presumably by exocytosis of synaptic vesicles which contain a high concentration of ATP as a common constituent.^{9),10)}

The possible involvement of ecto-protein kinase in synapse formation provides an attractive molecular mechanism for the selective stabilization of the most heavily used synaptic inputs, from which a considerable amount of ATP is released by high frequency stimulation.¹¹⁾ This sort of morphological plasticity at synapses, which can persist for a relatively long period, might be an important process in the establishment of long-term memory in the brain.²⁾ It would also be a new type of molecular mechanism in which biological information is transmitted not by the binding of agonist to receptors but by a supply of "messenger substrate" activating specific ecto-enzymes.

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