Cold stimulation evokes exocytotic vesicle release from PC12 cells

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(Received 5 September 2016; and accepted 21 September 2016)

ABSTRACT

The mechanism of peripheral blood circulation failure by low temperatures, known as Raynaud's phenomenon, is not well understood at present. We examined the possibility that the efferent fibers of peripheral sympathetic neurons may release transmitters directly by cold sensation. PC12 cells, a culture model of sympathetic neurons, were placed at 25°C for 30 min, fixed with the tannic acid-glutaraldehyde-osmium method and observed by electron microscopy. The number of omega-shaped exocytotic vesicle figures increased significantly compared with that in the control (37°C), although it was lower than that of the positive control (high potassium stimulation at 25°C and 37°C). As it is known that PC12 cells and sympathetic neurons do not express the typical cold sensing channels (TRPA1 and TRPM8), our results suggest that the peripheral sympathetic neurons may have an unknown cold monitoring system which reacts to low temperatures, release transmitters by exocytosis and directly regulate local blood circulation.

Thermoregulation is an important process in mammals. Changes in environmental temperature are monitored mainly by the sensory neurons of the dorsal root ganglia (8). The hypothalamus integrates the neural information and the peripheral autonomic nervous system regulates the body temperature by tuning the cutaneous blood circulation (7). The local mechanism of peripheral blood circulation failure by low temperatures, known as Raynaud's phenomenon, has been not well elucidated at present (7).

The molecular system of sensing temperature in mammals is based on the transient receptor potential (TRP) ion channel family (5, 12). Among them, only TRPA1 and TRPM8 channels are thought to be sensors of cold, both of which are known to exist in the sensory neurons of dorsal root ganglia. A study of calcium ion imaging revealed that relatively large numbers of peripheral sympathetic neurons are responsible for cold, although TRPA1 and TRPM8 channels are absent in those neurons (9).

Up to date, there has been no direct evidence that peripheral sympathetic neurons sense the cold stimuli and release their transmitters without input from the afferent sensory system. The aim of this study is to detect the exocytotic transmitter release in PC12 cells, a culture model of sympathetic neurons, after cold stimulation under electron microscopy. It is difficult to detect exocytotic figures in neuro-endocrine cells as their processes are extremely fast. Rapid freeze fixation is a useful and excellent method, but it needs a special apparatus and is unsuitable for a quantitative analysis. Tannic acid-glutaraldehyde-osmium fixation is known to preserve exocytotic figures relatively well, especially those of cored vesicles including bioamines and neuropeptides (1–4).

PC12 cells were sub-cultured with 10% FBS–Dulbecco's modified Eagle's medium at 37°C in a 5% CO₂ incubator. Cells were inoculated in culture dishes at 10⁶ cells/cm² and then placed in a 25°C incubator for 30 min. For the control (no treatment), the culture dishes were placed at 37°C for 30 min. For the positive control (depolarization), cells were stimulated with a high potassium and calcium solution (120 mM KCl, 115 mM NaCl, 6 mM CaCl₂) for 1 min at 25°C or 37°C. Thereafter, cells were quickly fixed with the tannic acid-glutaraldehyde-osmium

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Fig. 1 Electron micrographs of exocytotic vesicle release in PC12 cells by cold stimulation. The contents of exocytosed vesicles are seen as dense deposits by the effects of tannic acid-glutaraldehyde-osmium fixation (arrows).

method (1–4), dehydrated with a graded ethanol series and embedded in epoxy resin. Ultrathin sections were cut with an ultramicrotome (Leica Ultracut R) and mounted on copper grid meshes. After electron staining with gadolinium (EM stainer; Nissin EM Co. Ltd., Tokyo) and lead citrate solution, exocytotic figures were observed under an electron microscope (JEM1220; JEOL, Tokyo, Japan) at 80 kV. The number of omega-shaped figures were counted in 30 cells of each experimental group.

In this study, 25°C was used as the condition of cold stimulation because the typical cold sensing channels, such as TRPA1 and TRPM8, are known to open at this temperature (11, 12). After the cold treatment for 30 min, the omega-shaped vesicle figures could be detected by electron microscope (Fig. 1). In general, the contents of exocytosed vesicles are thought to disappear quickly by dissolving and diffusion. However, the tannic acid-glutaraldehyde-osmium fixation method effectively preserved the extracellular substances as electron dense structures including the contents of the exocytosed vesicles.

In the control cells (no treatment at 37°C), exocytotic figures were rarely seen. In the positive control (depolarization), high potassium and calcium ionic stimulation increased the number of exocytotic vesicle release not only at 37°C, but also at 25°C. We counted and compared the exocytotic figures in 30 cells of each experimental group.

The percentages of cells having exocytotic figures were as follows: 16.7% (37°C, no treatment), 80.0% (37°C, depolarization), 40.0% (25°C, no treatment) and 76.7% (25°C, depolarization) (Fig. 2). Statistically, the value at 25°C was significantly higher than that at 37°C control ($P < 0.05$, chi-square test). The

Fig. 2 The percentage of PC12 cells having exocytotic vesicle release. Cold stimulation (25°C for 30 min) significantly increased the percentage of cells containing exocytotic vesicles compared with the control (37°C, no treatment) ($P < 0.05$, chi-square test), while the either positive control (high K⁺ at 37°C and 25°C) certainly evoked exocytotic release compared with the control (no treatment at 37°C and 25°C, respectively).

average number of exocytotic figures per millimeter of cell membrane was as follows: 4.65 (37°C, no treatment), 36.7 (37°C, depolarization), 9.69 (25°C, no treatment) and 27.8 (25°C, depolarization) (Fig. 3).

These results indicate that PC12 cells, a model of sympathetic neurons, may release transmitters directly by vesicle exocytosis under cold stimulation. Currently, there is no information about the expression of typical cold sensing channels (TRPA1 and TRPM8) in PC12 cells. Only TRPV4, a known heat sensor, has been shown to be expressed in PC12 cells (6). A recent calcium imaging study, however, found that the neurons of the superior cervical ganglia that have no TRPA1 or TRPM8 respond to cold

Fig. 3 The number of exocytotic vesicle release per millimeter of cell membrane. Cold stimulation (25°C for 30 min) tended to increase the frequency of exocytotic vesicle release two-fold compared with the control (37°C). The positive control (high K⁺) evoked exocytotic release at an almost similar level at 25°C and 37°C.

stimuli, but not to menthol (9), although another study detected TRPA1 mRNA assessed by RT-PCR in the neurons of superior cervical ganglia (10).

In our experiments of depolarization, the exocytotic reactions of PC12 cells were almost similar at 25°C and 37°C. This suggests that the voltage- and calcium-dependent exocytotic machinery of PC12 cells may work almost normally at 25°C as well as at 37°C. There has been little information about the cold-sensing system of peripheral efferent neurons compared with that of afferent sensory neurons of the dorsal root ganglia (12). PC12 cells seem to be an excellent model for studying molecular and ultrastructural mechanisms of sensing low temperatures.

In summary, the present study demonstrated the exocytotic vesicle release in PC12 cells by cold stimuli, and suggests that the peripheral sympathetic

neurons directly control local blood circulation by sensing cold, independently to the central regulatory system.

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