Intracellular calcium and arachidonic acid increase SNAP-25 expression in cultured rat hippocampal explants, but not in cultured rat cerebellar explants

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Abstract

The effects of the increase of intracellular calcium, induced by membrane depolarization with 50 mM KCl, and arachidonic acid (AA) on the expression of 25-kD synaptosomal-associated protein (SNAP-25) were studied in cultured rat hippocampal and cerebellar explants, and PC12 rat pheochromocytoma cells, using immunoblot analysis. Incubation periods of 24 h and 48 h in 50 mM KCl increased SNAP-25 levels in hippocampal explants and PC12 cells, but not on cerebellar explants. Otherwise, a 24 h incubation with 10 μM AA increased SNAP-25 expression only in hippocampal explants, although 100 ng/ml phorbol 12-myristate 13-acetate (PMA) did not have effect. These results indicate that intracellular calcium and AA can modulate the expression of SNAP-25, depending on the origin of the tissue.

Keywords: 25-kD synaptosomal-associated protein; Hippocampus; Cerebellum; PC12 cells; Protein expression; Calcium; Arachidonic acid; Explants

Neuronal activity not only regulates the acute release of neurotransmitters, but it can also mediate the long-term adaptive responses by producing changes in gene expression. A variety of neurotransmitters that induce membrane depolarization can produce transient changes in intracellular Ca^{2+} concentrations which then lead to the activation of gene expression. For example, the activation of the NMDA (*N*-methyl-D-aspartate) subtype of glutamate receptor induces a rise in postsynaptic Ca^{2+}, which modulates gene expression [1]. The rise of intracellular Ca^{2+} induced by exposing neurons to high KCl concentration also increases gene expression [1].

The activation of the NMDA receptor also induces the release of arachidonic acid (AA) [14], which can also be produced by the joint activation of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptor and metabotropic glutamate receptors [7]. It has been demonstrated that AA can modulate the c-fos and Egr-1 mRNA in 3T3 fibroblasts [6] and c-jun mRNA in vascular smooth muscle cell [12]. Additionally, AA has been proposed as a retrograde messenger during long-term potentiation [10].

Neurotransmitters are released from a nerve terminal by exocytosis of synaptic vesicles. Biochemical studies of membrane fusion reactions have revealed proteins which may be important for membrane docking and/or fusion during exocytosis. These proteins include the synaptic vesicle protein synaptobrevin and the plasma membrane proteins syntaxin and 25-kD synaptosomal-associated protein (SNAP-25) [15,16]. When synaptic vesicles dock at release sites of the presynaptic plasma membrane, these proteins form a complex that in turn binds the cytoplasmic proteins *N*-ethylmaleimide-sensitive factor (NSF) and SNAP (soluble NSF attachment protein) [15,16]. The membrane proteins are referred to as SNAREs (SNAP-receptors). SNAP-25 is one of the SNARE proteins that is essential for exo-

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cytosis of synaptic vesicles, as indicated by its being a specific substrate for botulinum neurotoxins A and E, metalloproteases which effectively block neurotransmitter release [2,3].

In the present study, we studied the effect of 50 mM KCl and 10 μM AA on the expression of SNAP-25 in different tissue cultures: cultured rat hippocampal and cerebellar explants, and PC12 rat pheochromocytoma cells. Our hypothesis is that intracellular Ca^{2+} and/or AA can induce long-term changes in the presynaptic efficacy by regulating the expression of proteins involved in the secretion process.

Hippocampus and cerebellum were dissected from 6-day-old Sprague–Dawley rats, which were freed of meninges and washed in a solution containing 6.5 mM Na_2HPO_4, 3.4 mM NaH_2PO_4, 8 g/l NaCl, 4.1 mM KCl, 10 mM glucose, pH 7.4. Briefly, the tissue was chopped in explants of approximately of 1 mm^3, washed several times in a Dulbecco's modified Eagle's medium/F-12 mixture (1:1) (Sigma), supplemented with 5% fetal calf serum, 5% heat-inactivated horse serum, 100 μg/ml streptomycin and 100 U/ml penicillin and placed into 35-mm plates precoated with 100 μg/ml poly-L-lysine. After 1 h of incubation at 37°C the adhesion was completed and 1.5 ml of the same medium was added. The cultures were incubated at 37°C in a humidified 5% CO_2 95% air atmosphere. Hippocampal and cerebellar explants were treated 2 days after they were cultured.

PC12 rat pheochromocytoma cells, obtained from the American Type Culture Collection, were cultured in RPMI 1640 (Sigma) supplemented with 10% heat-inactivated horse serum (GIBCO), 5% fetal bovine serum (GIBCO), 100 μg/ml streptomycin and 100 U/ml penicillin. The cells were incubated at 37°C in humidified atmosphere of 5% CO_2 in air.

An isosmotic solution (170 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM HEPES, pH 7.4) was added to the culture medium to obtain a final concentration of 50 mM KCl. In control conditions, NaCl was substituted for KCl. In cultures treated with AA (Sigma), ethanol concentration was 0.05% and in cultures treated with phorbol 12-myristate 13-acetate (PMA) (Sigma), dimethyl sulfoxide concentration was 0.005%. Controls had the same concentrations of ethanol or dimethyl sulfoxide.

Control or treated cells in culture plates were lyzed in 1% Triton, 50 mM Tris (pH 7.4), 5 mM EDTA, 190 mM NaCl, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 10 μg/ml Pepstatin and 400 μg/ml DNase I, mixed with 50 mM Tris–HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol and 0.1% bromophenol blue, and boiled for 2 min. Total cell proteins were separated by SDS-PAGE in 12% polyacrylamide and electrophoretically transferred (32 V for 4 h) to nitrocellulose 0.45 μm membranes (Novex). SNAP-25 was detected using a monoclonal anti-SNAP-25 antibody (Sternberger Monoclonals) and a secondary antibody labeled with alkaline phosphatase. The immunoblot was developed with bromochloroindol phosphate/nitro blue tetrazolium as substrate.

To compare changes in the expression of SNAP-25, the blots were scanned with a Genius scanner and analyzed by a software described by Celedon et al.[4]. The relationship between different amounts of SNAP-25 and the area of each band given by the software, gave a correlation index of 0.989 (data not shown). Data are expressed as means ± SEM of the percentage of the increase in the levels of SNAP-25, compared to controls.

Data were analyzed by *t*-test. Differences were considered significant if $P < 0.05$.

Changes in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) after K^+ depolarization were measured in Indo-1 loaded single cells, according to Grynkiewieicz et al. [8] using a fluorescence inverted microscope (Diaphot-200, Nikon) described by Nassar-Gentina et al. [11].

In order to study the effect of the increase of $[Ca^{2+}]_i$ on SNAP-25 levels we first evaluated the effect of a depolarizing solution containing 50 mM KCl in single Indo-1-loaded cells. As shown in Fig. 1, 50 mM KCl generated a rapid and transient increase in $[Ca^{2+}]_i$ either in hippocampus and cerebella cultured cells, and PC12 cells. Therefore, we evalu-

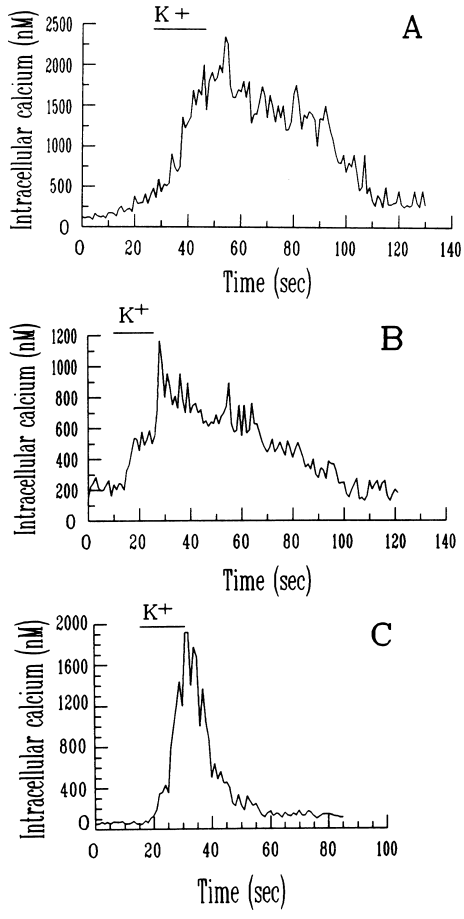

Fig. 1. Increase in $[Ca^{2+}]_i$ induced by high K^+ in hippocampal (A), cerebellar (B) and PC12 cells (C). $[Ca^{2+}]_i$ signals were generated by a depolarizing solution containing 50 mM KCl, which was applied for 15 s in single Indo-1-loaded cells.

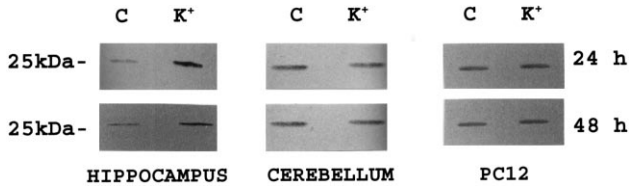

Fig. 2. Representative immunoblot analysis of SNAP-25 expression in hippocampal explants, cerebellar explants and PC12 cells incubated for 24 or 48 h in control conditions (C) or in 50 mM KCl. Control or treated cultures were lyzed and 90 μg of protein were subjected to 12% SDS-PAGE, blotted and probed as described in the text.

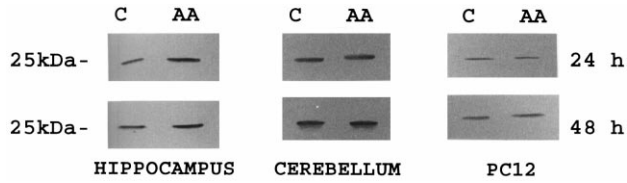

Fig. 3. Representative immunoblot analysis of SNAP-25 expression in hippocampal explants, cerebellar explants and PC12 cells incubated for 24 or 48 h in control conditions (C) or in 10 μM AA. Control or treated cultures were lysed and 90 μg of protein were subjected to 12% SDS-PAGE, blotted and probed as described previously.

ated the effect of different periods of incubation with 50 mM KCl in the culture medium.

Incubation periods of 12 h with 50 mM KCl did not affect the levels of SNAP-25 in hippocampal explant cultures, but a 24 h incubation period with high KCl induced an increase of the SNAP-25 levels (Fig. 2), reaching 151 ± 23% ($n = 3$, $P < 0.005$) above the protein levels in control cultures (Table 1). As shown in Fig. 2, the elevation in the SNAP-25 levels in hippocampal cultures was maintained after 48 h incubation period with 50 mM KCl, reaching 115 ± 30% ($n = 3$, $P < 0.05$) over the SNAP-25 levels in control cultures (Table 1).

No effect was observed when cerebellar explant cultures were incubated for different periods of time with 50 mM KCl (Fig. 2).

In PC12 cells, incubation periods of 24 h induced an increase of SNAP-25 levels of 29.5 ± 4% ($n = 5$, $P < 0.001$) above the protein levels in control cells (Table 1). As shown in Fig. 2, the elevation in SNAP-25 levels in PC12 cells was maintained after 48 h incubation period with 50 mM KCl, reaching 33.8 ± 4% ($n = 5$, $P < 0.001$) over the protein levels in control cells (Table 1).

Hippocampal and cerebellar explant cultures and PC12 cells were also incubated for different periods of time with 10 μM AA in the culture medium. After 12 h of incubation with 10 μM AA no effect was observed in the SNAP-25 levels of hippocampal explant cultures, but a 24 h incubation period with AA induced an increase of the SNAP-25 expression (Fig. 3), reaching 150 ± 22% ($n = 3$, $P < 0.005$) over the protein levels in untreated cultures (Table 1). After a 48 h incubation period with 10 μM AA, the elevation in the SNAP-25 levels in hippocampal cultures was smaller, 65 ± 14% ($n = 4$, $P < 0.05$) above the protein levels in control cultures (Table 1).

No effect was observed on the SNAP-25 levels when cerebellar explant cultures were incubated for different periods of time with 10 μM AA. Further, no effect of AA was observed in PC12 cells (Fig. 3).

Since reportedly AA increases gene expression by protein kinase C (PKC) activation [6], we evaluated the effect of phorbol 12-myristate 13-acetate (PMA) on SNAP-25 levels in hippocampal explants. No significant effect was observed when the explant cultures were incubated for different periods of time with 100 ng/ml PMA (data not shown), suggesting that the effect of AA on SNAP-25 expression in hippocampal explants would not be mediated by activation of PKC. However, some isoforms of PKC are not regulated by phorbol esters [5,17]. Conversely, AA can regulate gene expression independently of PKC. Indedd, Shu et al. [13] have reported that the increase of pro-enkephalin mRNA induced by AA is partially mediated by a Ca^{2+}/calmodulin pathway, but by not protein kinase A or protein kinase C pathways.

Taken together, the present results show that SNAP-25 expression can be regulated by the increase of $[Ca^{2+}]_i$ and AA. However, this effect depends on the origin of the tissue. We do not have plausible explanations for the lack of effect of high K^+ or AA on SNAP-25 expression in cerebellar explants, nor for the lack of effect of AA on PC12 cells, but we believe it is an important subject for future studies.

Finally, our results yield two important findings: (1) cell stimulation that determine increases of $[Ca^{2+}]_i$ or AA production, can regulate the expression of proteins involved in the secretion process, such as SNAP-25, which could be

Table 1

Effect of high KCl and AA on SNAP-25 levels in hippocampal explants, cerebellar explants and PC12 cells

Type of culture	% of increase of SNAP-25 levels	
	50 mM KCl	10 μM AA
Hippocampal explants		
Control	0 ± 16% ($n = 3$)	0 ± 15% ($n = 3$)
After 24 h	151 ± 23%** ($n = 3$)	150 ± 22%** ($n = 3$)
After 48 h	115 ± 30%* ($n = 3$)	65 ± 14%* ($n = 4$)
Cerebellar explants		
Control	0 ± 10% ($n = 3$)	0 ± 11% ($n = 3$)
After 24 h	2 ± 9% ($n = 3$)	2 ± 8% ($n = 3$)
After 48 h	3 ± 11 ($n = 3$)	2 ± 10% ($n = 3$)
PC12 cells		
Control	0 ± 1% ($n = 4$)	0 ± 2% ($n = 4$)
After 24 h	29.5 ± 4%*** ($n = 5$)	3 ± 1% ($n = 3$)
After 48 h	33.8 ± 4%*** ($n = 5$)	4 ± 2% ($n = 3$)

Cell cultures were incubated for 24 h or 48 h in control conditions or in the presence of 50 mM KCl or 10 μM AA. SNAP-25 levels was determined by immunoblot analysis as described previously. Each value represents means ± S.E.M. of the percentages of the increase in the levels of SNAP-25. The number of experiments is indicated in parentheses.

*$P < 0.05$, **$P < 0.005$, ***$P < 0.001$ versus each control.

followed by an increase in the presynaptic efficacy and (2) this effect on the expression of synaptic proteins would not occur in the cerebellum, where long-term depression has been reported [9].

The authors wish to thank Mr. Neftalí Cárdenas for his technical assistance. This study was supported by Grant 1960352 from Fondecyt.

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