V-1, a catecholamine biosynthesis regulatory protein, positively controls catecholamine secretion in PC12D cells

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Abstract **Stably transfected PC12D cell lines overexpressing a catecholamine biosynthesis regulatory protein, V-1, were used to examine the functional role of V-1 in catecholamine secretion. High K^+-induced dopamine secretion in V-1 overexpressing clones was shown to be markedly potentiated compared with control clones carried with a vector alone. As assayed intracellular calcium concentration ($[Ca^{2+}]_i$) using fura-PE3, V-1 overexpression was observed to enhance high K^+-elicited $[Ca^{2+}]_i$ elevation. Electron microscopic analysis revealed an increase in dense-cored vesicle formation by V-1 overexpression. These results suggest that the enhancement of high K^+-induced dopamine secretion by V-1 overexpression results from the potentiation of high K^+-induced $[Ca^{2+}]_i$ elevation and the increase in the number of dense-cored vesicles.**

Key words: V-1; Overexpression; PC12D cells; Dopamine biosynthesis; Dopamine secretion; Exocytosis

1. Introduction

Abnormalities in catecholamine biosynthesis and neurotransmission have been implicated as the causes of neurological and psychiatric diseases [1]. Therefore it is of great importance to elucidate the molecular mechanisms that control catecholamine biosynthesis and neurotransmission. However the mechanisms are not fully understood. We have recently established stable transfectants that overexpress V-1 using the catecholamine-producing neuronal cell line PC12D and have demonstrated that V-1 functions in the control of the gene expression of catecholamine biosynthesizing enzymes to promote catecholamine biosynthesis [2]. V-1 is a cytosolic protein consisting of 117 amino acids that contains 2.5 tandem repeats of the cdc10/SWI6 motif, also known as the ankyrin repeat [3,4]. This motif has been demonstrated to be crucial for protein–protein interactions between various ankyrin repeat proteins with multiple functions and the specific partner proteins [5,6]. The expression of V-1 ubiquitously occurs in adrenal chromaffin cells as well as catecholaminergic neurons of the peripheral and central nervous systems (the PNS and CNS) [2], although this protein is expressed with a different quantity among catecholaminergic cells in a transmitter phenotype-dependent manner. We here describe the first evidence that V-1 participates in the promotion of catecholamine secretion from PC12D cells.

2. Materials and methods

2.1. Generation of PC12D cell clones that stably overexpress V-1 protein

The PC12D cell subclone of rat pheochromocytoma cells (PC12) was cultured as previously described [7]. V-1 overexpressing and vector control clones were established as previously reported [2].

2.2. Dopamine (DA) content and secretion assays

DA content and DA secretion were measured as described previously [2,8]. For DA secretion assay, 1×10^6 cells were plated on 35-mm tissue culture dishes and harvested 48 h later. Cells were washed twice with low K^+ buffer (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15 mM HEPES–Tris, pH 7.4), and then incubated for 10 min with high K^+ buffer (85 mM NaCl, 60 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15 mM HEPES–Tris, pH 7.4) to evoke membrane depolarization. The incubation buffer was collected and centrifuged at $800\times g$ for 10 min to remove detached cells. The resultant supernatant was acidified with perchloric acid (final concentration 0.4 N). Cellular DA was extracted with 0.4 N perchloric acid. The amounts of DA in the incubation buffer and cell extracts were quantified by high performance liquid chromatography–enhanced chemiluminescence detection as previously described [2]. The DA secretion was expressed as a percentage of the total DA stored in the cells.

2.3. Measurements of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

Cells were plated at a density of 5×10^5 cells per 35-mm dish and cultured overnight, and subjected to the measurements of $[Ca^{2+}]_i$ according to the previously reported method [9] except for using fura-PE3/AM. For loading fura-PE3, cells were washed with Hanks' HEPES buffer and incubated in 2 ml of Hanks' HEPES buffer containing 10 μM fura-PE3/AM for 1 h at 37°C. Subsequently, cells were resuspended in 1 ml of this buffer without fura-PE3/AM. Fura-PE3-Ca^{2+} signal was measured with a spectrofluorometer (CAF-100, Jasco, Tokyo, Japan): excitation light having wavelengths of 340 nm and

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380 nm, and emission of 520 nm (37°C). At the end of experiments, the maximum and minimum fluorescence levels were determined with 0.1% Triton X-100 and 5 mM ethyleneglycol bis(β-aminoethyl)-*N*′,*N*′-tetraacetic acid (EGTA, pH 8.2), respectively, and the $[Ca^{2+}]_i$ was calculated by the use of the fura-PE3-Ca^{2+} dissociation constant of 290 nM as reported by Vorndran et al. [10].

2.4. Electron microscopy

Cells (1×10^6) were plated on 60-mm tissue culture dishes. Following cell culture for 3 days, cells were washed by ice-cold Hanks' HEPES buffer (137 mM NaCl, 0.4 mM KCl, 0.4 mM $MgSO_4$, 1.3 mM $CaCl_2$, 5.6 mM glucose, 0.5 mM $MgCl_2$ 10 mM HEPES–NaOH, pH 7.5), detached from culture dish by pouring the same buffer, and collected by centrifugation. The resultant cell pellets were fixed by 2% glutaraldehyde–4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 1 h at 4°C, and post-fixed in 2% osmium tetroxide in the same buffer for 1 h at 4°C. The fixed pellets were embedded in 1.5% Type VII agarose (Sigma) solution with low melting point by cooling on ice. After trimming, agarose blocks containing cell pellets were dehydrated with a graded series of ethanol and embedded in Epoxy resin, routinely. Ultrathin sections were cut, stained with 5% uranyl acetate and Raynolds' lead citrate solution, then examined with a transmission electron microscope (JEOL JEM 1200EX, Jeol Ltd.).

2.5. Western blot analysis

5×10^5 cells were plated on 35-mm tissue culture dishes and cultured for 48 h. Cells lysates were prepared as previously reported [2]. Protein samples were electrophoresed on SDS–polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Millipore). The blotted membrane was blocked and then incubated successively with anti-SNAP-25 (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein-25) antibody (Calbiochem; 1:1000) overnight at 4°C and horseradish peroxidase conjugated goat anti-rabbit IgG (1:2000) for 1 h at room temperature. The membrane was reprobed with anti-protein kinase Aα (as internal control) antibody (Sanata Cruz Biotechnology), and signals were visualized with enhanced chemiluminescence detection reagents (Pierce). The immunoreactive signals were quantified using a densitometer (ATTO Densitograph).

3. Results

DA contents in V-1 overexpressing clones at the resting state were markedly higher than those in control clones (Fig. 1A), which was very similar to the previously reported data [2], and the DA level of PC12D cells was 46–64% of those of two V-1 overexpressing clones (data not shown). To evaluate the effect of V-1 overexpression on catecholamine secretion, we examined high K^+(60 mM)-induced DA secretion from control and V-1 overexpressing clones. As shown in Fig. 1B, the depolarization-induced DA secretions were appreciably augmented in comparison with those from control clones. DA secretion from V-1 overexpressing clones was also shown to be elevated compared with that from PC12D cells (data not shown).

The transmitter secretion/release from neurons and neuroendocrine cells is regulated by Ca^{2+}. Ca^{2+} not only triggers the final step of transmitter secretion/release, but also is involved in many other events which are important for vesicle recycling [11–16]. To investigate the mechanism by which V-1 overexpression causes the enhancement of DA secretion from PC12D cells, the increasing effect of high K^+ on $[Ca^{2+}]_i$ was compared between V-1 overexpressing clones and control clones, using a calcium indicator of fura-PE3. All cell lines tested exhibited an initial transient increase in $[Ca^{2+}]_i$ followed by a sustained phase (Fig. 2A). The high K^+-evoked increases in the initial transient and sustained $[Ca^{2+}]_i$ phases were observed to be potentiated in V-1 overexpressing clones, when compared with those in control clones (Fig. 2A,B). In addition, the resting levels of $[Ca^{2+}]_i$ were a little higher in V-1 overexpressing clones than in control clones (Fig. 2A).

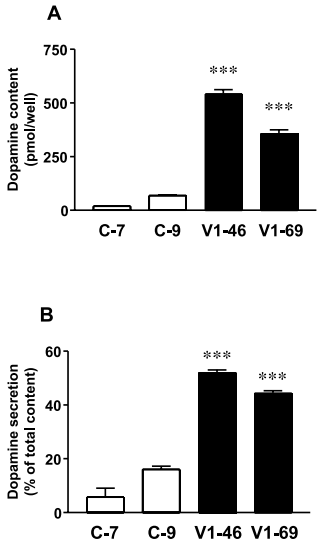

Fig. 1. Effects of V-1 overexpression on DA contents and high K^+-induced DA secretion. A: Cellular contents of DA. Values indicate the means ± S.E.M. ($n=5$). Asterisks denote significant ($P<0.001$) from control clones. B: Secreted DA evoked by high K^+. B: Cells were incubated with high K^+ buffer for 10 min. The amount of DA was quantified as previously reported [2]. The amount of DA secreted is expressed as a percentage of the total cellular DA content. Values indicate the means ± S.E.M. ($n=5$). Significant differences from two vector control clones are indicated at $P<0.001$ (Student's *t*-test). C-7, C-9: control clones; V1-46, V1-69: V-1 overexpressing clones.

Catecholamine are stored in the dense-cored vesicles containing vesicular monoamine transporter-1 in PC12 cells [17]. To evaluate the effect of V-1 overexpression on dense-cored vesicle formation in PC12D cells, ultrathin sections of V-1 overexpressing clones and the parental PC12D cells were analyzed by using a transmission electron microscope. The electron microscopic examination showed that there exist dense-cored vesicles with various sizes in both the assayed parental cells and V-1 overexpressing clones (Fig. 3A–D). In the cytoplasm of the parental PC12D cells, a limited number of small dense-cored vesicles with moderate electron opacity were observed (Fig. 3A). These vesicles were mostly round in shape, and namely 90% (62/69) of them had the long/short axis ratio of less than 1.5, while the remaining vesicles exhibited the long/short axis ratio ranging between 1.5–1.67 (data not shown). As shown in Fig. 3D, the length of the long axis of these vesicles observed in the parental cells ranged between 80 and 140 nm. On the other hand, V-1 overexpressing clones largely included oval dense-cored vesicles with the long/short axis ratio of larger than 1.5, among which the vesicles with the long/short axis ratio of larger than 1.8 were frequently observed. But the round dense-cored vesicles in these clones composed a smaller part compared with the parental cells: the round vesicles occupied 38.6% (17/44) and 30% (18/60) in clone V1-46 and clone V1-69, respectively. Furthermore the long axis of the dense-cored vesicles in the V-1 overexpressing clones exhibited wider distribution than that observed in the parental cells (Fig. 3D). The long axes of the dense-cored vesicles were 105 ± 15.2 nm (the mean ± S.D., $n=69$), 128 ± 37.7 nm ($n=60$) and 128 ± 27.8 nm ($n=60$) in

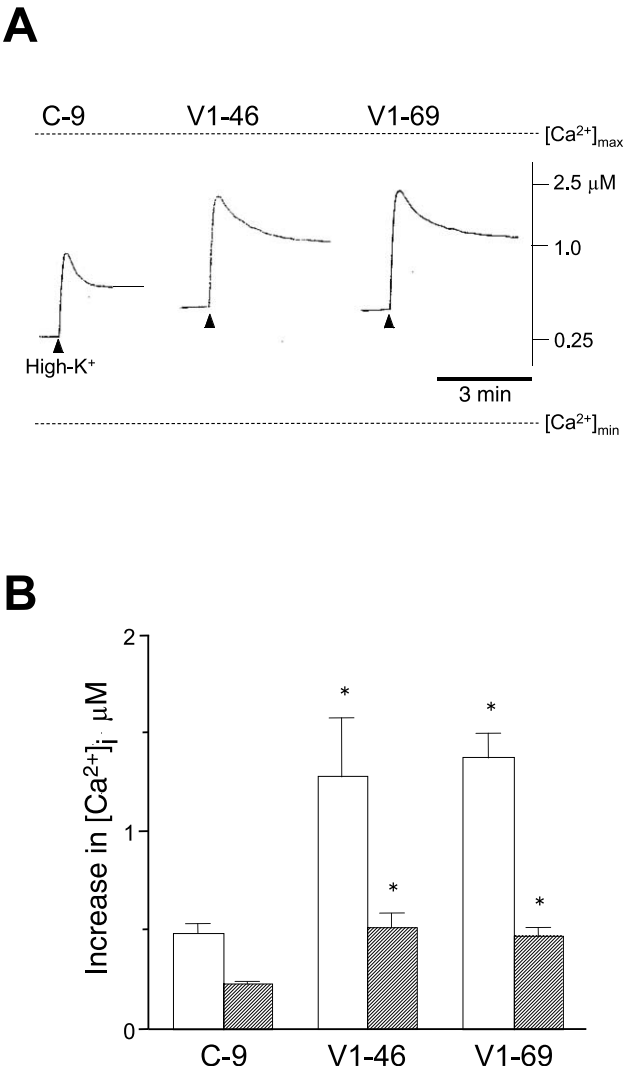

Fig. 2. High K^+-induced increase in $[Ca^{2+}]_i$ of control clone (C-9) and V-1 overexpressing clones (V1-46 and V1-69). A: High K^+ (60 mM) was administered at closed triangles. $[Ca^{2+}]_{max}$ represents the maximal $[Ca^{2+}]_i$ level in the presence of 0.1% Triton X-100, and $[Ca^{2+}]_{min}$ for the minimal level in the presence of 5 mM EGTA. B: Statistical representation for high K^+-induced increase in $[Ca^{2+}]_i$. In the ordinate, increased amounts of transient (white column) and slow phases (hatched column) represent the amounts of initial transient phase and slow phase at 3 min after exposure to high K^+, respectively. Values are expressed as the means ± S.E.M. ($n = 5$–6). Asterisks represent the significant difference from levels of C-9 with $P < 0.05$: F-test followed by Student's t-test or Aspin–Welch test.

the parental cells, clone V1-46 and clone V1-69, respectively. Differences in the means of the length of the long axes between the parental cells and clones V1-46 or V1-69 were statistically significant ($P < 0.01$) by the Cochran–Cox t test. The dense-cored vesicles in V-1 overexpressing clones showed high electron opacity within themselves. The size and electron opacity of the dense-core observed in the V-1 overexpressing clones appeared to resemble the catecholamine-containing vesicles found in the adrenal medullary cells of the rat (data not shown). In addition large dense-cored vesicles were observed mainly in the peripheral region of the cytoplasm of the V-1 overexpressing clones, and frequently within several hundred nanometers from the plasma membrane (data not shown).

SNAPs are key protein in exocytosis. SNAP-25 (synaptosomal-associated protein of 25 kDa) is necessary for exocytosis [12,14,15,18,19,20]. Thus the expression level of SNAP-25 in V-1 overexpressing clones and control clones was examined by Western blot analysis. Western blot analysis showed that expression of SNAP-25 in the V-1 overexpressing clone was reduced to approximately 50% of that in the control clones (Fig. 4).

4. Discussion

In the present study, we provide the first evidence that V-1 overexpression enhances high K^+-induced DA secretion in PC12D cells. This study also revealed an enhancement of high K^+-induced increase in $[Ca^{2+}]_i$ in V-1 overexpressing clones. It is well known that exocytosis occurs through a pathway including secretory vesicle recruitment, priming, and docking, followed by Ca^{2+}-triggered membrane fusion. In chromaffin cells, the secretory vesicle recruitment is an ATP- and Ca^{2+}-dependent step, although it can also be activated by an alternative Ca^{2+}-independent pathway [14]. Therefore the stimulation of high K^+-induced increase in $[Ca^{2+}]_i$ in V-1 overexpressing clones appears to account for an increment in high K^+-induced DA secretion from these clones.

We have earlier reported that V-1 overexpressing clones show the increments in expression of mRNA coding for tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase and DA β-hydroxylase, resulting in augmentations of DA and norepinephrine production [2]. Furthermore we have recently reported an appreciable increase in the expression of GTP cyclohydrolase I (GCH) which is the rate-limiting enzyme for the biosynthesis of tetrahydrobiopterin, known as an essential cofactor for TH, in V-1 overexpressing clones [21]. This increase in GCH gene expression has been shown to result from an enhancement of cyclic AMP-responsive element-dependent transcription [21]. These findings demonstrate that V-1 controls transcription of multiple genes responsible for catecholamine biosynthesis to promote catecholamine production in PC12D cells. In addition, since the acute TH activation after nerve stimulation is mediated by Ca^{2+}/calmodulin-dependent protein kinase and protein kinase C [22], the small elevation of the resting $[Ca^{2+}]_i$ in V-1 overexpressing clones compared with control clones (Fig. 2A) may contribute to the stimulation of catecholamine biosynthesis via TH. The increase of cellular DA contents by V-1 overexpression in PC12D cells was confirmed in this study (Fig. 1A), which is quite consistent with our data previously reported [2]. It has been recently reported that produced catecholamine are stored in the dense-cored vesicles containing vesicular monoamine transporter1 in PC12 cells [17]. Indeed, in this study, it was evidently shown that two independent V-1 overexpressing PC12D clones contained small dense-cored and large dense-cored vesicles much more than the parent PC12D cells. These morphological observations are consistent with the apparent increase of catecholamine contents in the two V1 overexpressing clones compared with those of the parent PC12D cells. Accordingly it is quite possible that the increase of catecholamine production by V-1 results in the promotion of the large and small dense-cored vesicle formation. Therefore these observations suggest that V-1 increases the number of large and small dense-cored vesicles and potentiates high K^+-induced

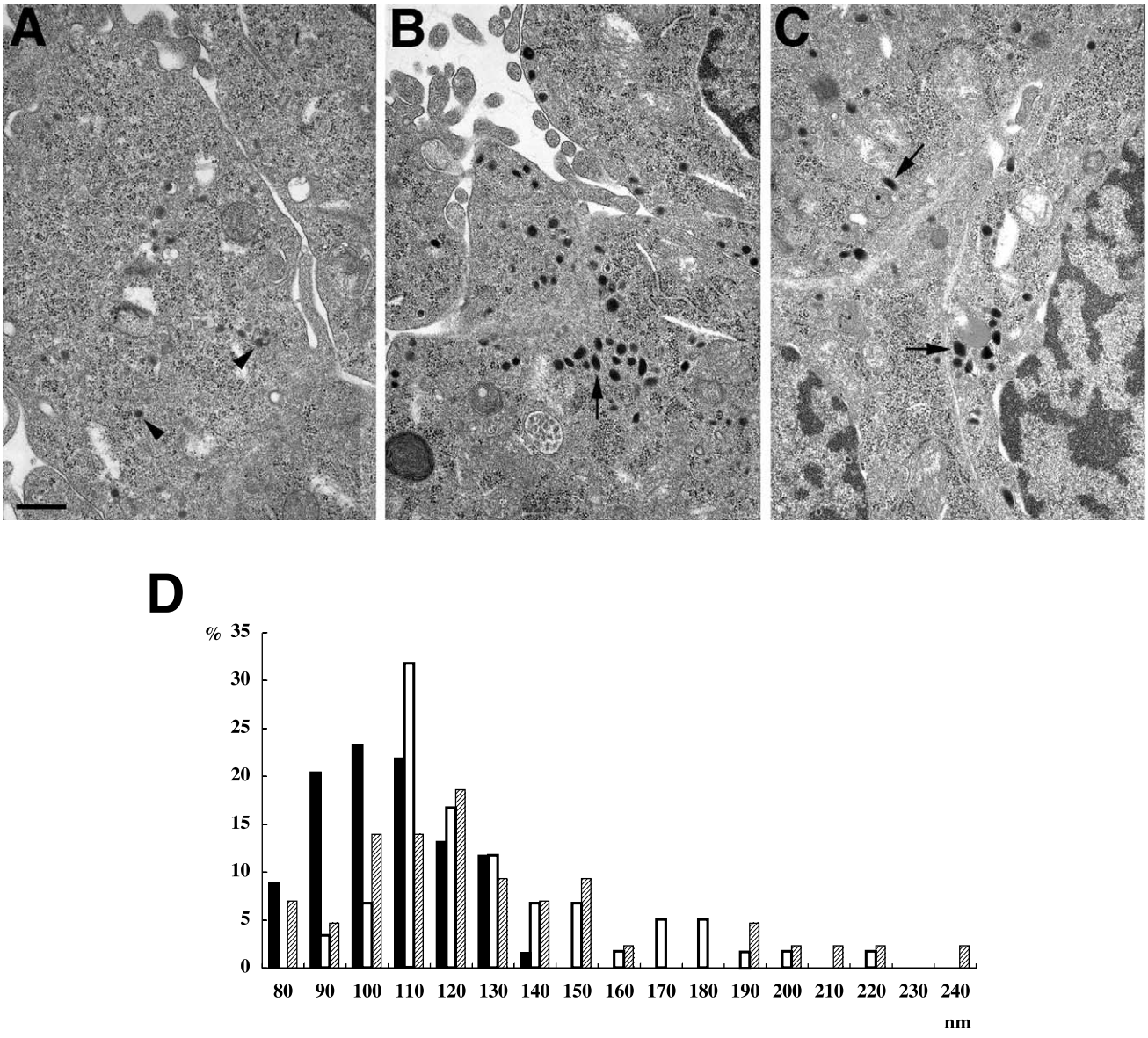

Fig. 3. Electron microphotographs showing ultrastructural features of parental PC12D cells (A) and V-1 overexpressing clones, V1-46 (B) and V1-69 (C). A: A limited number of small dense-cored vesicles are seen (arrowheads). B,C: Besides the small dense-cored vesicles, large dense-cored vesicles are also observed (arrows). Bar, 500 nm. D: A histogram of the distribution of the long axes of dense-cored vesicles. Black, white and hatched columns indicate % ratio of the long axes of dense-cored vesicles in parental PC12D cells, V1-46 and V1-69, respectively. Figures of the abscissa indicate central values of the long axes of grouped dense-cored vesicles. For example, the columns for 120 indicate % ratio of the long axes between 115 and 125 nm.

$[Ca^{2+}]_i$ elevation to enhance the depolarization-induced DA secretion.

It is usually difficult to substantiate how catecholamine overproduction affects the molecular mechanism that controls the gene regulation of the enzymes responsible for the biosynthesis and secretion, because catecholamine-producing cells exhibit a transient increase of catecholamine biosynthesis in response to physiological stimuli, including neurotransmitters. A unique characteristic of V-1 overexpressing clones is that catecholamine overproduction is stably sustained. It is a very important finding in the present study that in V-1 overexpressing clones, the upregulation of gene expression of enzymes involved in catecholamine biosynthesis [2] coincides with the downregulation of gene expression of SNAP-25 (Fig. 4), but not synapsin Ia and Ib, which are regulator proteins of neurotransmitter secretion/release [13,14,23] (unpublished data). An attractive interpretation of this finding is that there exists the mechanism that coordinately controls the regulation of genes responsible for catecholamine biosynthesis and its release at the transcription level to maintain an appropriate catecholamine level in vivo.

The expression of SNAP-25 was observed to be reduced in V-1 overexpressing clones. Type E botulinum toxin has been shown to cleave SNAP-25 to completely block the secretion from PC12 cells [18,19], indicating that SNAP-25 is essential for the secretion in PC12 cells. However, unexpectedly the reduced expression of SNAP-25 in V-1 overexpressing clones did not exert a decreasing effect on DA secretion. Recently

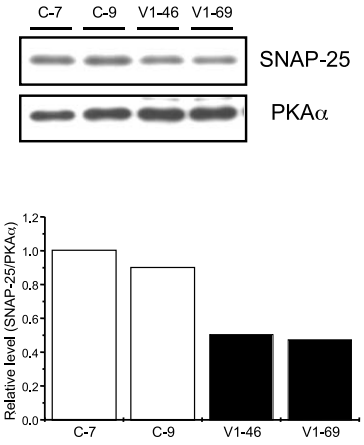

Fig. 4. Downregulation of SNAP-25 protein expression in V-1 overexpressing clones. Cell lysates (5 μg of protein) from V-1 overexpressing clones (V1-46 and V1-69) and control clones (C-7 and C-9) were separated on 18% SDS–PAGE for Western blot analysis. Shown are representative data of two independent experiments (upper panel). SNAP-25 protein expression in both V-1 overexpressing clones and control clones was normalized to protein kinase Aα protein expression assayed for each sample (lower panel).

SNAP-25-deficient mice have been generated by gene targeting [20]. It has been shown that the CNS neurons of SNAP-25 null mutant mice fail to exhibit high K^+-evoked exocytosis, whereas in the heterozygous mutants showing an approximately 50% reduction of SNAP-25 protein expression, exocytosis is detectable after high K^+-induced depolarization [20]. Therefore the findings regarding SNAP-25 expression in the V-1 overexpressing clones raises the possibility that the stimulation of high K^+-induced increase in $[Ca^{2+}]_i$ might overcome the decreasing effect of this downregulation of SNAP-25 expression. We are currently testing the possibility.

V-1 gene is widely expressed in the PNS and CNS neurons and the adrenal medulla chromaffin cells [2,24]. Taken together with the morphological observations, the results of this study suggest that V-1 positively controls neuronal functions, such as catecholamine biosynthesis and secretion/release in the PNS and CNS as well as adrenal chromaffin cells.

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