The Ca$^{2+}$ channel $\beta_2$ subunit is selectively targeted to the axon terminals of supraoptic neurons

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**Abbreviations:** MNC, magnocellular neurosecretory cell; NH, neurohypophysis; OT, oxytocin; SON, supraoptic nucleus; VP, vasopressin

Voltage-gated Ca$^{2+}$ (Ca$\alpha$) channels mediate the influx of Ca$^{2+}$ that triggers exocytotic release in both neurons and neuroendocrine cells. The physical relationship between the channels and the exocytotic machinery is a crucial determinant of activity-secretion coupling and therefore the mechanisms by which specific types of Ca$\alpha$ channels are localized to neuronal and neuroendocrine release sites are critically important for exocytotic secretion. The mechanisms by which Ca$\alpha$ channels are targeted and anchored to axonal release sites are incompletely understood.

Ca$\alpha$ channels are composed of a complex of subunits. The Ca$\alpha$$\beta$ subunit forms the ion selective pore and determines most of the channel properties. Ten types of Ca$\alpha$$\beta$ subunits have been identified and grouped into 3 families, which are called Ca$\alpha$1, Ca$\alpha$2, and Ca$\alpha$3 and mediate L-type Ca$^{2+}$ currents, P/Q-, N-, and R-type Ca$^{2+}$ currents, and T-type Ca$^{2+}$ currents, respectively. High voltage-activated Ca$^{2+}$ channels (those in the Ca$\alpha$1 and Ca$\alpha$2 families) require a Ca$\alpha$ subunit in the channel complex. There are 4 types of Ca$\beta$ subunits (Ca$\beta_1$$\cdots$Ca$\beta_4$) and the presence of specific subunits in the complex can influence channel properties such as inactivation. All 4 types are expressed in mammalian brain, but co-immunoprecipitation experiments have shown that the large majority of L-type, N-type, and P/Q-type channels in the brain are associated with either Ca$\beta_1$ or Ca$\beta_4$, and only a very small fraction is constructed with Ca$\beta_2$. The Ca$\beta_2$ subunit is not widely expressed in the brain, but is found in specific brain areas such as the hippocampus, thalamus, and cerebellum. RT PCR experiments suggest that Ca$\beta_2$ is the dominant Ca$\beta$ subunit in the hippocampus and co-immunoprecipitation experiments suggest that a significant fraction of the Ca$\alpha$$\beta_2$ subunit in PC12 neuroendocrine cells is associated with Ca$\beta_2$. Ca$\beta$ subunits are important in mediating the targeting of Ca$\alpha$ to the plasma membrane, but their roles, if any, in targeting Ca$\alpha$ to specific sites in neurons is unclear. The co-expression of different Ca$\alpha$$\beta_1$ and Ca$\beta$ subunits in polarized epithelial cells suggested that targeting is determined solely by the Ca$\alpha$$\beta_1$ subunit in the case of Ca$\alpha$1.2 and Ca$\alpha$2.2 channels, but that the associated Ca$\beta$ subunit could determine the targeting of Ca$\alpha$2.1 channels. Ca$\beta_2$ and Ca$\beta_4$ have been reported to be distributed in clusters localized to synapses when exogenously expressed in cultured hippocampal neurons and exogenously expressed Ca$\beta_2$ was reported to localize in the axon terminals of cultured hippocampal neurons, suggesting that Ca$\beta$ subunits might be targeted to and anchored at release sites. In contrast, a study of the distribution of fluorescently labeled Ca$\beta$ subunits expressed in cultured hippocampal neurons found that all Ca$\beta$ subunits accumulate in synaptic terminals, which led the authors to conclude that Ca$\beta$ subunits do not possess targeting properties.

The magnocellular neurosecretory cells (MNCs), which are responsible for the release of the hormones oxytocin and vasopressin into the circulation, provide a unique opportunity to study subcellular expression of Ca$\beta$ subunits. While MNC somata are found in 2 specific nuclei in the hypothalamus, the supraoptic nucleus (SON) and the paraventricular nucleus, neuroendocrine release occurs from MNC swellings and endings...
somata and terminals. Differences in the volume of vasopressin and/or oxytocin. Comparisons of evoked currents in MNC somata and terminals have suggested that N- and P/Q-type currents in the terminals inactivate more rapidly and more completely than those in the somata and that the P/Q-type currents are less sensitive to block by ω-agatoxin IVA. Since the association of different CaV subunits can alter the rate of inactivation of both N- and P/Q-type Ca2+ currents and can also alter the sensitivity of P/Q-type currents to ω-agatoxin IVA, the observed differences in biophysical properties could be due to differences in the CaV subunits associated with CaV subunits in the subcellular locations.

We used immunoblot analysis to compare the expression of CaV subunits in the neurohypophysis, SON, and whole brain. We found that all 4 CaV subunits are expressed in all of these tissues, but when we compared the densities of the bands, we found that the ratio of expression in the neurohypophysis and SON was significantly greater for the CaV subunit than for any other CaV subunit. This suggests that the fraction of the total CaV that is expressed in the neurohypophysis is greater than that of the other CaV subunits. We also used immunohistochemistry on slices of the SON and neurohypophysis to observe immunoreactivity to CaV subunits in the MNC terminals and in the SON somatodendritic region and found that the ratio of staining intensity again suggested that CaV is enriched in MNC terminals relative to the MNC somata. Our results support the hypothesis that CaV is selectively targeted to MNC axon terminals. This targeting could play an important role in the targeting of Ca2+ channel α subunits to MNC terminals and in determining their biophysical properties.

Results

Immunoblots probed with CaV antibodies showed that all of the 4 CaV subunits are expressed in whole brain, neurohypophysis, and SON (Fig. 1A). The presence of multiple bands is likely to reflect both alternative splicing of the CaV mRNA and post-translational modification of the CaV subunits. We noted that the total intensities of all the bands for CaV were roughly similar in the SON and NH, whereas the bands for the other CaV subunits were markedly more intense in the SON. We therefore measured the total intensity for all of the bands for each CaV antibody in each tissue and used these values to determine the ratios of the total band intensity between different tissues. This comparison was performed in 4 sets of experiments, one of which is displayed in Figure 1A. Figure 1B is a bar graph showing the means ± SDs obtained by comparing the ratios for the four sets of experiments. A repeated measures analysis of variance shows that the difference between the ratio for CaV and for each of the other CaV subunits was highly significant (P < 0.01), whereas none of the other CaV subunits were significantly different from each other. These data demonstrate that the fraction of the total CaV subunit that is present in the neurohypophysis is higher for the CaV subunit than it is for any of the other CaV subunits.

We also noted a difference in the pattern of bands for the CaV subunit in the neurohypophysial and SON tissues.
compared with those in the whole brain. A comparison of the staining patterns for Caβ2 shows that the highest molecular weight band found in the whole brain homogenate is absent from the lane showing the staining for the neurohypophysial tissue. This band was very faint in the SON and was undetectable when we used isolated MNC somata (data not shown), suggesting that this variant is not expressed in MNC somata or terminals. A possible interpretation of this observation and its implications will be discussed below.

The immunoblot data suggest that the MNC terminals have relatively more Caβ2 than tissue from either whole brain or the SON. It is possible, however, that this is simply a reflection of the high levels of that particular Caβ2 subunit in the MNCs and that the lower level in the SON reflects a greater proportion of Caα channels from non-MNC elements. We therefore used immunohistochemical techniques to probe slices of SON and neurohypophysis using the same Caβ2 subunit antibodies. We co-stained the slices with a mixture of antibodies directed against neurophysin I, which labels only oxytocin-containing MNCs, and neurophysin II, which labels only vasopressin-containing MNCs, to allow positive identification of MNC somata and dendrites in the SON and MNC terminals in the neurohypophysis. We have shown previously that this combination of neurophysin antibodies effectively distinguishes MNC elements in the neurohypophysis from both the surrounding astrocytes (which are selectively labeled by an antibody directed against the glial marker S100β) and synaptic inputs into the neurohypophysis (which are labeled by an antibody directed against synaptophysin). The results of these experiments are shown in Figure 2. An example of the immunohistochemical results for Caβ2 are shown in Figure 2A, with the SON results shown in the top row of images and the results for the neurohypophysis in the bottom row of images. Examples of results for the other three Caβ subunit antibodies are not shown in the interests of space, but were analyzed in the same manner. In each row the first image (labeled “NP”) shows immunoreactivity for the mixture of the two neurophysin antibodies, the second image (labeled “β2”) shows the immunoreactivity for the Caβ2 antibody, and the third image (labeled “NP+β2”) shows the overlay of the two previous images. The first image was used to make a binary mask such that pixels showing immunoreactivity for neurophysin were given a value of 1 and others were given a value of 0, and this binary mask was multiplied by the second image to make the fourth image (labeled as “β2/NP+”), which shows the immunoreactivity for Caβ2 only in those areas that are neurophysin-positive, i.e., the MNC somata and dendrites in the SON image and the MNC terminals in the neurohypophysial image. These images were used to calculate the mean intensity of staining for each of the Caβ2 subunits in the MNC somata and dendrites and in the MNC terminals. The results shown in Figure 2B represent the means (± SEMs) of the results from 7–14 slices obtained from 4 rats. The immunoreactivity for each of the Caβ2 subunit antibodies was higher in the terminals, which probably reflects the higher density of Caα channels in the MNC terminals. To determine whether there were differences in the extent to which any of the Caβ subunits were targeted to the MNC terminals.

Figure 2. Immunoreactivity for the Caβ2 subunit suggests that it is selectively targeted to MNC terminals. (A) Immunohistochemical dual labeling results for antibodies directed against the Caβ2 subunit and the neurophysins. The results for the SON are shown in the top row of images and the results for the neurohypophysis (NH) are shown in the bottom row of images. The 2 rows of images show, respectively, the immunoreactivity to the neurophysin antibodies (NP), the immunoreactivity to the Caβ2 antibody (β2), the overlay of these 2 images (NP+β2) and the immunoreactivity for the Caβ2 antibody in locations that were positive for neurophysin (β2/NP+). See text for a description of how this analysis was performed. The scale bar indicates 20 μm. (B) A bar graph showing the mean ± SEM of the average intensities of immunoreactivity to antibodies directed against the indicated Caβ2 antibodies determined in 7–14 slices of either the SON or the neurohypophysis (NH) prepared from 4 rats. (C) Images of slices of the neurohypophysis (NH) showing the immunoreactivity to each of the 4 Caβ2 antibodies normalized to the mean value of immunoreactivity for that same antibody in MNC somata and dendrites in the SON. Note that the normalized staining for Caβ2 is the brightest of the four. (D) A bar graph showing the mean ± SD of the average intensity of immunoreactivity to each of the 4 Caβ2 antibodies in 7–14 slices of the neurohypophysis (NH) normalized to the mean value of immunoreactivity to that antibody in MNC somata and dendrites in the SON. The ratio for the Caβ2 subunit is greater than the ratios for the other 3 Caβ subunits (P < 0.01).
we normalized the Caβ subunit immunoreactivity in each slice of the neurohypophysis by dividing them by the mean intensity of immunoreactivity for that Caβ subunit in all of the SON slices. The images in Figure 2C show examples of the immunoreactivity for the four Caβ subunits normalized in this fashion. Note that the image for Caβ1b is far brighter than any of the other subunits. The bar graph in Figure 2D shows the mean ratios (± SD) of the immunoreactivities for MNC axon terminals to that of the MNC somatodendritic regions for each of the Caβ subunits. An analysis of variance test shows that the ratio for Caβ1b is higher than for the other Caβ subunits and that this difference is highly significant (P < 0.01). The difference in immunoreactivity in MNC somata and dendrites vs. MNC terminals suggests that Caβ1b is specifically targeted to the terminals.

Discussion

Caβ subunits contribute to the biophysical properties of Caα channels, and the differences observed between specific types of currents in the soma and terminals of MNCs16 could therefore be due in part to differences in the associated Caαβ subunits. It is also possible that specific Caβ subunits could be involved in the targeting of Caα channels to MNC terminals. We therefore undertook studies to determine whether any of the four Caβ subunits were expressed at different levels in the MNC somata and axon terminals. We probed immunoblots of tissue homogenates of SON and neurohypophysis with antibodies directed against the Caβ subunits and found that there is a significantly higher ratio for the total intensity of Caβ1b between neurohypophysis and SON than for any of the other Caβ subunits. We also tested the hypothesis of specific targeting of Caαβ to MNC terminals by performing immunohistochemistry on slices of SON and neurohypophysis. These studies allowed us to look specifically at the labeling in MNC somata and MNC terminals by comparing the Caβ subunit immunoreactivity only in areas that were immunoreactive for the neurophysins. These experiments showed that Caβ1b has a greater ratio of staining in the MNC terminals to staining in the MNC soma than the other Caβ subunits, which therefore suggests that Caβ1b is selectively targeted to the MNC terminals.

We observed in our immunoblots that the largest molecular weight isoform of Caβ1a appears to be absent from the MNCs. Although we have not identified the molecular identity of this variant, one intriguing possibility is that it represents the palmitoylated form of Caβ1a. Caβ1a is the only known Caβ variant that can be palmitoylated4 and the palmitoylated form of Caβ1a has a molecular weight of 72 kilodaltons,14 which is the approximate size of the band that is present in the whole brain but absent in the neurohypophysis (Fig. 1A). Although the association of Caβ1a causes slowing of Ca current inactivation, most other variants of Caβ actually accelerate inactivation, and the presence of palmitoyl groups is likely to be necessary for this slowing of inactivation.4 The absence of the Caβ1a subunit would therefore be consistent with the observation that Ca2.1 and Ca2.2 inactivate quickly in MNC terminals.15,16 An alternative explanation for the difference in Ca2.1 current inactivation in MNC somata and terminals would be the differential targeting of splice variants of the Ca2.1α subunit. We have reported that the MNCs appear to express one or both of 2 variants of Ca2.1 that lack portions of the intracellular linker sequence between channel domains I and III,15 which includes the synaptic protein interaction (synprint) site.3 The variant with the larger deletion was shown in a heterologous expression system to have a marked rightward shift in the voltage dependence of inactivation compared with the full-length channel or to the other variant, but we have no evidence for selective targeting of these splice variants within the MNCs. A point mutation in the intracellular I-II loop of the α4 subunit of Ca2.1 that regulates current inactivation has been identified in mammalian brain, as well as a mutation in an extracellular sequence that alters the sensitivity of the channels to ω-agatoxin IVA.30 Differential targeting of variants containing these mutations could contribute to differences in Ca2.1 currents in MNC somata and terminals, but we have no evidence for expression of these variants in MNCs.

It has been proposed by Obermair et al.14 that the selective targeting of Caβ subunits to neuronal terminals depends on their association with specific Caα4β subunits that are themselves selectively targeted and this could form an alternative hypothesis for the targeting of the Caβ subunit to MNC terminals. These authors suggested that Caβ1b may be slightly more likely to associate with somatodendritic Ca1.2 channels whereas Caβ2 may be more likely to interact with presynaptic Caα channels. This mechanism does not appear to explain the enrichment of Caβ1 in MNC terminals, however, since patch clamp experiments have been used to demonstrate that 60–70% of the Caα current in MNC terminals is carried by N- and P/Q-type Ca2 channels,24 which are the Caα types most prevalent in synaptic terminals,1 and the total fraction of current mediated by L-type current in MNC terminals (about 25%24) is similar to the fraction observed in MNC somata.21 The preferential association of specific Caβ subunits with specific Caαβ subunits does not therefore appear to be a likely explanation for the enrichment of Caβ1 in MNC terminals.

Our data suggest that Caβ1b may have a role in targeting or anchoring Caα channels in the axonal terminals of the MNCs. It is not clear if this property is unique to MNCs or neuroendocrine terminals or may also be involved in neurons that express Caβ subunits, such as hippocampal neurons.9,12 Our data are consistent with the observation that Caβ may be targeted to axon terminals in cultured hippocampal neurons,1 but differs because in these cells Caβ1b was involved in clustering of Caα channels at synaptic sites, whereas MNC terminals do not have synapses and Caα channels appear not to be clustered.26,34

In conclusion, our findings suggest that the Caβ1 subunit is enriched in MNC terminals and that a higher fraction of Caα channels in the MNC terminals are constructed with this subunit than are Caα channels in the SON. Furthermore, our immunohistochemical results suggest that the Caβ1 subunit is targeted to MNC terminals, because the ratio of immunoreactivity in the terminals to that in the somatodendritic region is higher for Caβ1 subunit than for the other Caβ subunits. These data
suggest that the Ca$_{\beta_3}$ subunit may play a role in targeting Ca$_\alpha$ channels to the MNC terminals. Ca$_\alpha$ channels in the terminals are primarily responsible for the activation of exocytotic secretion of the neurohormones vasopressin and oxytocin and their construction with the Ca$_{\beta_2}$ subunit may have important implications for activity-secretion coupling.

**Experimental Procedures**

This work was approved by the University of Saskatchewan’s Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

**Immunoblotting**

Male Long-Evans rats (200–300 g) were anesthetized with halothane and killed with a rodent guillotine. Brain slices that included most of the supraoptic nuclei of the hypothalamus were cut and blocks that included a portion of the 2 supraoptic nuclei were excised. Care was taken to ensure that the blocks contained as much SON tissue and as little non-SON tissue as possible. The neurohypophysis was removed and the intermediate lobe was cut away using dissecting scissors. Tissues from the supraoptic nuclei, neurohypophysis, and the rest of the rat brain were then homogenized, solubilized and separated using standard electrophoretic techniques.
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