Journal of Bioenergetics and Biomembranes, Vol. 30, No. 4, 1998

Metabolism and Trafficking of N-Type Voltage-Operated Calcium Channels in Neurosecretory Cells

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Received April 30, 1998

The N-type voltage-operated calcium channel has been characterized over the years as a high-threshold channel, with variable inactivation kinetics, and a unique ability to bind with high affinity and specificity ω-conotoxin GVIA and related toxins. This channel is particularly expressed in some neurons and endocrine cells, where it participates in several calcium-dependent processes, including secretion. ω-conotoxin GVIA was instrumental not only for the biophysical and pharmacological characterization of N-type channels but also for the development of *in vitro* assays for studying N-type VOCC subcellular localization, biosynthesis, turnover, as well as short-and long-term regulation of its expression. We here summarize our studies on N-type VOCC expression in neurosecretory cells, with a major emphasis on recent data demonstrating the presence of N-type channels in intracellular secretory organelles and their recruitment to the cell surface during regulated exocytosis.

KEY WORDS: Calcium channel; neuron; endocrine; N-type; conotoxin; secretion; vesicle; granule; recruitment.

INTRODUCTION

The N-type calcium channel was first defined as a novel, neither-T nor L-type, voltage-operated calcium channel (VOCC) subtype in sensory neurons (Nowycky *et al.*, 1985; Fox *et al.*, 1987). It was suggested to have a steep voltage dependency of activation, to activate at lower voltages than the L-type, to display a faster inactivation, and to be completely inactivated at relatively positive holding potentials (Nowycky *et al.*, 1985; Fox *et al.*, 1987). Oversimplification of these first reports led many authors to define as "N-type" any fast inactivating component of macroscopic, high-threshold calcium currents. It was later realized, however, that N-type channels could not be so easily discriminated from other high-threshold calcium channels by only using these variable biophysical criteria (Bean, 1989; Swandulla *et al.*, 1991; Miller, 1992). In particular, the N-type VOCCs have been shown to display different degrees of inactivation, to be still available at relatively positive holding potentials, and to activate at higher positive voltages than the L-type (Aosaki and Kasai, 1989; Plummer *et al.*, 1989; Carbone *et al.*, 1990; Kasai and Neher, 1992; Boland *et al.*, 1994; Elmslie *et al.*, 1994). The variable inactivation kinetics of the N-type could be due to the presence of different gating modes (Plummer and Hess, 1991; Rittenhouse and Hess, 1994), the expression of different molecular isoforms (Lin *et al.*, 1997), or, possibly, the differential association with accessory beta subunits (Scott *et al.*, 1996).

Instrumental for the "new" definition of the N-type VOCC was ω-conotoxin GVIA (ω-Ctx GVIA),

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0145-479X/98/0800-0399$15.00/0

a peptide toxin extracted from the venom of the fish hunting snail *Conus Geographus* (Kerr and Yoshikami, 1984; Rivier *et al.*, 1987). First described as a potent blocker of both L-type and N-type VOCCs in neurons (Nowicky *et al.*, 1985; Fox *et al.*, 1987; McCleskey *et al.*, 1987; Hirning *et al.*, 1988), this toxin was later shown *to spare* the L-type and to be a highly selective and irreversible blocker of only N-type VOCCs (Sher *et al.*, 1988; Carbone *et al.*, 1990; for a review see Sher and Clementi, 1991).

The N-type VOCC was first supposed to be expressed only in neurons. However, due to the use and selectivity of ω-Ctx GVIA (more recently supported by molecular biology approaches) it was later recognized that many other secretory cells (previously thought to express only L-type VOCCs) did also express N-type VOCCs. These include pheochromocytoma cell lines and normal adrenal chromaffin cells (Sher *et al.*, 1988; Plummer *et al.*, 1989; Jan *et al.*, 1990), insulinoma and normal pancreatic beta cells (Sher *et al.*, 1992; Pollo *et al.*, 1993; Ramanadham and Turk, 1994), small-cell lung carcinoma cells (DeAizpurua *et al.*, 1989; Sher *et al.*, 1990), anterior pituitary cells (Suzuki and Yoshioka, 1987; Lievano *et al.*, 1994), thyroid C cells (Dubel *et al.*, 1992), and testicular Sertoli cells (D'agostino *et al.*, 1992).

The combined availability of both different neuronal and endocrine cell lines expressing N-type VOCCs, and of a highly selective ligand such as ^{125}I-ω-Ctx GVIA, was again instrumental for approaching the *in vitro* study of the metabolism of this channel under physiological and pathological condition.

BIOCHEMISTRY, MOLECULAR BIOLOGY, AND REGIONAL DISTRIBUTION

The N-type VOCC was shown to be composed, similarly to the L-type, of several polypeptides, in particular 210–250 kDa alpha$_1$ subunits, a 140 kDa alpha$_2$ subunit, and one or more beta subunits (Abe and Saisu, 1987; Cruz *et al.*, 1987; Yamaguchi *et al.*, 1988; Barhanin *et al.*, 1988; Marqueze *et al.*, 1988; Ahlijanian *et al.*, 1991; McEnery *et al.*, 1991; Sakamoto and Campbell, 1991; Westenbroek *et al.*, 1992; Witcher *et al.*, 1993; Leveque *et al.*, 1994), although other molecular weight constituents have occasionally been described by only some of the above authors.

In all neuronal and endocrine cells where it is expressed, the N-type VOCC mediates at least part of the influx of calcium that triggers neurotransmitter and hormone release (Sher and Clementi, 1991; Olivera *et al.*, 1994). In line with this crucial role in secretion, the N-type VOCC physically interacts, in both neurons and endocrine cells, with other proteins of the "SNARE" complex, such as syntaxin, synaptotagmin, and synaptobrevin (Bennet *et al.*, 1992; David *et al.*, 1993; Leveque *et al.*, 1994; ElFar *et al.*, 1995). Furthermore, the G-protein mediated modulation of the gating and permeability of N-type VOCCs is a well-characterized phenomenon (Schultz *et al.*, 1990; Kasai, 1991; Pollo *et al.*, 1992; Kuo and Bean, 1993; Hille, 1994; Ikeda, 1996; Herlitze *et al.*, 1996); in line with this, a co-purification of N-type VOCCs with the G_o subunit was recently obtained (McEnery *et al.*, 1994).

The alpha$_{1B}$ subunit, coding for the N-type VOCC, was cloned from human (Williams *et al.*, 1992), rat (Dubel *et al.*, 1992), rabbit (Fujita *et al.*, 1993), and mouse (Coppola *et al.*, 1994) neuronal tissues. Different isoforms of alpha$_{1B}$ are expressed in different tissues and can be co-expressed in the same neuron. Recently, Lipscombe and colleagues showed that an alpha$_{1B}$ isoform containing a "SFMG" tetrapeptide sequence is particularly expressed in rat brain, while an isoform lacking the "SFMG" sequence but containing an "ET" dipeptide sequence is more expressed in sympathetic ganglia (Lin *et al.*, 1997). The differential expression of N-type VOCC isoforms could represent the basis for some discrepancy found in the literature on the biophysical properties of N-type VOCCs.

Antibodies against the alpha$_{1B}$ protein, antibodies against ω-conotoxin GVIA, and probes for the alpha$_{1B}$ mRNA (Northern blotting and *in situ* hybidization) have been recently used to map the distribution of N-type VOCCs in different areas of the nervous system (Fortier *et al.*, 1991; Westenbroek *et al.*, 1992; Dubel *et al.*, 1992; Fujita *et al.*, 1993; Volsen *et al.*, 1995; Tanaka *et al.*, 1995). The results confirmed essentially those previously obtained with ^{125}I-ω-Ctx GVIA binding assays (Barhanin *et al.*, 1988; Dooley *et al.*, 1988; Wagner *et al.*, 1988) and autoradiography (Kerr *et al.*, 1988; Takemura *et al.*, 1988; Maeda *et al.*, 1989), showing a wide expression of N-type VOCCs throughout the brain, with the highest density in cerebral cortex, hippocampus, olfactory bulb, hypothalamus and thalamus, and cerebellar cortex.

CELLULAR LOCALIZATION

In line with its important contribution to neurotransmitter release, the N-type VOCC has been shown

to reside mainly presynaptically. Immunolocalization studies revealed, for example, that N-type VOCCs are clustered at the presynaptic active zones of the frog neuromuscular junction (Robitaille *et al.*, 1990; Cohen *et al.*, 1991; Torri-Tarelli *et al.*, 1991). In hippocampal neuronal cultures, N-type VOCCs cluster at sites of synaptic contacts (Jones *et al.*, 1989), and in chick ciliary ganglia, they are clustered on the presynaptic membrane of the calix (Stanley and Atrakchi, 1990; Haydon *et al.*, 1994). In nerve growth factor-treated PC12 cells cultures, N-type VOCCs are accumulated in the growing neurite processes (Reber and Reuter, 1990; Usowicz *et al.*, 1990).

However, in mammalian neuromuscular junctions, where the P/Q-type VOCC plays a dominant role in the control of transmittion, the N-type VOCC is preferentially localized in nerve terminal-associated Schwann cells (Day *et al.*, 1997). A minor presence on the nerve terminals, however, cannot be excluded on the basis of immunolocalization data only. In fact, the presence of N-type VOCCs in mammalian neuromuscular junctions is suggested by some reports showing ω-Ctx GVIA effects also in these sites (Rossoni *et al.*, 1994).

A "postsynaptic" role of N-type VOCCs has also been suggested on the basis of a postsynaptic, dendritic immunolocalization found in many neurons (Mills *et al.*, 1994), and by the presence of N-type calcium currents in "dendrosomes" (Kavalali *et al.*, 1997). N-type VOCCs on dendrites are supposed to participate in the amplification and integration of both synaptic events and back-propagating action potentials.

Worth noting, however, is the complementary localization of L-type and N-type VOCCs in most neurons, where the L-type is mainly localized on the cell bodies and at the bases of major dendrites (Westenbroek *et al.*, 1990) while the N-type is distributed all along the dendritic tree and in a subpopuation of dendritic spines (Westenbroek *et al.*, 1992; Mills *et al.*, 1994).

Finally, a "cell body/leading process" localization of N-type VOCCs has been demonstated in migrating cerebellar granule cells; at these sites, the N-type VOCC plays a crucial role in allowing the influx of calcium that permits granule cell migration (Komuro and Rakic, 1992). Granule cell dedrites continue to express large amounts of N-type VOCCs also in the mature animals, as evidenced by autoradiography studies on *weaver* mutant mice completely lacking cerebellar granule cells (Maeda *et al.*, 1989).

N-TYPE VOCC METABOLISM

The characterization of the cellular and molecular mechanisms underlying the developmentally regulated, highly polarized, and often clustered expression of the N-type VOCCs is a challenging task for neurobiologists. The identification of cell lines expressing endogenous N-type VOCCs (Sher *et al.*, 1988, 1990, 1992) and the availability of an irreversible ligand such as ω-Ctx GVIA has helped in the development of *in vitro* systems to study N-type VOCC metabolism, as a first step toward the characterization of N-type VOCC cell biology.

The half-life of the N-type VOCC was found to be around 15–18 hours in different neuronal cell lines (Table I), as determined by "prelabeling" ^{125}I-ω-Ctx GVIA binding assays, "postlabeling" ^{125}I-ω-Ctx GVIA binding assays, and fura2 fluorimetric determinations (Passafaro *et al.*, 1992; Passafaro *et al.*, 1993). Both selective acid washes, differential temperature, and lysosomotropic drugs allowed independent measurements of the rates of channel "internalization" and channel "degradation"; we found the two rates to be very similar under physiological conditions.

Interestingly, the rate of N-type VOCC turnover was found to be *reduced*, in a time-dependent manner in cells exposed to differentiating agents for several days (Table I). This occurred independently from the specific agent used to induce cellular differentiation. This channel turnover "slow-down" is probably responsible for the increased expression of the alpha$_{1B}$ subunit in differentiated cells (Fig. 1), the "stabilization" of N-type VOCCs in the plasma membrane (Passafaro *et al.*, 1992), and the "up-regulation" of N-

Table I. N-Type VOCC Turnover in Different Neuronal Cell Lines[a]

Cell line	Control (half-life, hours)	Differentiated (half-life, hours)
IMR-32	16 ± 2	45 ± 6
PC-12	17 ± 2	32 ± 5
SH-SY5Y	16.5 ± 3	35 ± 2
F11	15 ± 3	27 ± 3

[a] N-type VOCC turnover was measured with the aid of ^{125}I-ω-Ctx GVIA as described in Passafaro *et al.* (1992, 1993). IMR-32, PC-12, SH-SY5Y, and F11 cells were exposed for 10 days to dibutyryl-cAMP and 5-BrdU, NGF, retinoic acid, and serum deprivation, respectively.

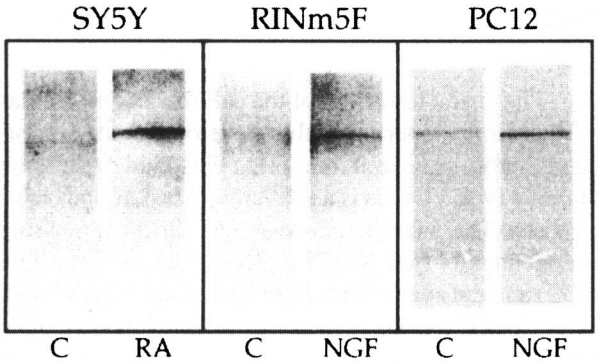

Fig. 1. Increased expression of the N-type VOCC alpha$_{1B}$ subunit in differentiated neurosecretory cells. Immunoblottings were performed as described in Passafaro *et al.* (1996) and McEnery *et al.* (1997), utilizing CW14, a selective anti-alpha$_{1B}$ affinity-purified antibody (McEnery *et al.*, 1997). C = control cells; RA = retinoic acid-treated cells; NGF = nerve growth factor-treated cells.

type currents in differentiated cells (Reber and Reuter, 1990; Carbone *et al.*, 1990; Toselli *et al.*, 1991).

N-type VOCC turnover can also be *increased*. We found that anti-calcium channel antibodies present in Lambert–Eaton myasthenic syndrome patients' sera not only immunoprecipitate N-type VOCCs (Sher *et al.*, 1989) [in parallel to the P/Q type VOCC (Motomura *et al.*, 1995)], but also rapidly stimulate N-type VOCC turnover when added to neuronal cells in culture (Passafaro *et al.*, 1992), a process leading to the previously demonstrated down-regulation of N-type VOCC expression after exposure to the patients' sera (Sher *et al.*, 1989; Leys *et al.*, 1991). For the moment we cannot compare the rates of N-type VOCC turnover with the rates of other VOCCs, since data on the latter are not available. A direct comparison of the turnover of the different channels in the same cellular systems will bring important new information.

A POOL OF RECRUITABLE N-TYPE VOCCS IN NEUROSECRETORY CELLS

Both neuronal and endocrine cell lines, as well as normal chromaffin cells, have recently been found to contain a large intracellular pool of N-type VOCCs. This pool can be recruited to the cell surface in a few hours if the cells are exposed to calcium channel antagonists (Passafaro *et al.*, 1994), or in a matter of a few minutes if the cells are exposed to secretagogues (Passafaro *et al.*, 1996). We summarize below the main characteristics of the two forms of N-type VOCC recruitment.

Antagonists-Induced Recruitment

When intact, living neuronal and endocrine cells were continuously exposed to pM concentrations of ^{125}I-ω-Ctx GVIA, the binding of the toxin to the cells at 37°C *did not* reach a plateau in a few minutes, as expected from data obtained on cell homogenates or membrane extracts (Sher *et al.*, 1988). Instead, the *specific* ^{125}I-ω-Ctx GVIA binding increased steadily for several hours, reaching a plateau only after 6–8 hours of incubation (Passafaro *et al.*, 1994; see also Marqueze *et al.*, 1988). With respect to the binding of ^{125}I-ω-Ctx GVIA detectable on intact cells at 4°C or 20°C, the binding of ^{125}I-ω-Ctx GVIA at 37°C was 400–600% higher (Table II). This huge increase in binding was not due to "unspecific" pinocytosis of the toxin. Furthermore, most of the "recruited" binding sites were present on the plasma membrane, as evidenced by their selective sensitivity to acid washes. This was the first suggestion that at 37°C some peculiar process was taking place with ^{125}I-ω-Ctx GVIA binding to living cells, and led to the idea that the toxin was able to "stimulate" its own binding site recruitment (Passafaro *et al.*, 1994).

More compelling evidence of the ability of ω-Ctx GVIA to "stimulate" N-type VOCC recruitment was obtained by showing that the continuous presence of the toxin was not necessary to see recruitment. In fact, experiments consisting of short pulses with a saturating concentration of unlabeled ω-Ctx GVIA, followed by extensive washes and variable chase periods at 37°C, demonstrated that while rapidly blocking the "preexisting" channels, the toxin caused a large amount of "new" channels to be recruited to the cell surface during the several hours of chase (Passafaro *et al.*, 1994).

Interestingly, this form of N-type VOCC recruitment was not dependent on protein synthesis, sug-

Table II. Omega-Conotoxin GVIA-Induced Recruitment of Surface N-Type VOCCS in Different Neurosecretory Cells[a]

Cell line	% of control
IMR32	555 ± 57
SH-SY5Y	464 ± 21
PC-12	610 ± 35
RINm5F	516 ± 32

[a] The recruitment of surface N-type VOCCs was determined as described in Passafaro *et al.* (1994) by exposing the cells to ^{125}I-ω-Ctx GVIA (25 pM) for 4–6 hours at 37°C.

gesting that pre-formed channels were involved. This does not mean that a specific transcriptional or translational regulation of N-type VOCC subunit expression cannot occur under different conditions (Cavalie *et al.*, 1994).

The intracellular origin of the newly recruited channels was finally confirmed by both "fixation/permeabilization" experiments, by temperature-dependent blockade of protein secretion, and by using drugs interfering with intracellular protein transport. In particular, most of the recruitment was blocked, reversibly, by Brefeldin A (BFA), a drug able to inhibit protein export from the Golgi apparatus (Rosa *et al.*, 1992), and by nocodazole, a microtubule depolymerizing agent.

A similar temperature- and BFA-sensitive recruitment of N-type VOCCs was also obtained with the reversible and inorganic VOCC blocker cadmium, suggesting that this process could be a common cellular reaction in response to VOCC blockade.

Since the newly recruited channels are functional as evidenced by both fura2 and patch clamp experiments (Passafaro *et al.*, 1994), it is likely that neuronal cells exposed to different classes of calcium antagonists could become "potentiated" in terms of calcium influx, after drug withdrawal.

Secretagogue-Induced Recruitment

More recently we have shown that N-type VOCC recruitment could be caused also by secretagogue agents (Passafaro *et al.*, 1996). With respects to the former type of recruitment caused by calcium antagonists, this latter form is rapid (minutes vs hours), smaller (around 100% increase), and BFA-insensitive. Nevertheless it also has an intracellular origin, as shown by both "fixation/permeabilization" and subcellular fractionation experiments.

In both human neuroblastoma, rat pheochromocytoma, and rat insulinoma cells, "regulated" secretion stimulated by either high potassium, the phorbol ester TPA, the calcium ionophore ionomycin, or high barium concentrations always produces N-type VOCC recruitment (Table III). In all the cell types, and with every stimulus, the dose-dependence and time course of hormone secretion match very closely those of N-type VOCC recruitment.

The legitimate doubt that this recruitment process could represent a specific feature of *in vitro* growing, tumoral, cell lines was ruled out by our recent demonstration that also in primary cultures of bovine chromaffin cells a secretagogue-induced recruitment of N-type VOCC does occur (Passafaro *et al.*, manuscript in preparation).

As in the case of calcium antagonists-induced N-type VOCC recruitment, the N-type VOCCs recruited by secretagogues are also functional as evidenced by fura2 and patch clamp experiments (Passafaro *et al.*, 1996; Passafaro *et al.*, manuscript in preparation), implicating this process in the "potentiation" of calcium signals and, in turn, of physiological and pathological calcium-dependent events.

What do we know about the identity of the secretory organelle(s) that contain the N-type VOCCs and mediate their recruitment? The strong parallelism between hormone (Chromogranin B and serotonin) secretion and N-type VOCC recruitment suggests a specific localization of N-type VOCCs in the membrane of the hormone-containing secretory granules themselves. This evidence is supported by some of our data showing that a peak of ^{125}I-ω-Ctx GVIA binding sites (Passafaro *et al.*, 1996) as well as of alpha$_{1B}$ immunoreactivity (Passafaro *et al.*, manuscript in prep-

Table III. Secretagogue-Induced Recruitment of Surface N-Type VOCCs in Different Neurosecretory Cells[a]

Cell line	KCl	TPA	Ionomycin	Barium
	(% of control)			
IMR-32	204 ± 3.6	218 ± 27	210 ± 17	ND
PC-12	212 ± 9.8	199 ± 9.5	198 ± 10.5	ND
RINm5F	175 ± 1.9	182 ± 1.4	ND	169 ± 6.2
Bovine adrenal chromaffin	173 ± 1.4	ND	ND	199 ± 2.4

[a] The values represent the increase in surface ^{125}I-ω-Ctx GVIA binding with respect to parallel control cells. The secretagogue-induced N-type VOCC recruitment was evaluated by exposing the cells to either KCl (50 mM), TPA (20 nM), ionomycin (100 nM), or barium (10 mM) for 15–30 min at 37°C as described in Passafaro *et al.* (1996). ND = not determined.

aration) is found in subcellular fractions of PC12 cells well separated from both the plasma membrane and the synaptophysin-containing microvesicles. On the other hand, we found a good co-sedimentation of the intracellular N-type VOCCs with the Chromogranin B-containing organelles (Passafaro *et al.*, 1996).

Further support for a secretory granule localization of at least part of the intracellular N-type channels comes from experiments showing that a "purified" preparation of bovine chromaffin granules contains both ^{125}I-ω-Ctx GVIA binding sites and alpha$_{1B}$ immunoreactivity. Finally, specific alpha$_{1B}$ immunoreactivity was found, at the immuno-gold E.M. level, on the membrane of some chromaffin granules (Passafaro *et al.*, manuscript in preparation, and Fig. 2).

The presence of N-type VOCCs in the membrane of secretory organelles adds complexity to the molecular machinery of secretion. In most models, the N-type VOCCs is only viewed as a *surface* protein, interacting with other membrane or vesicular proteins such as syntaxin, synaptotagmin, and synaptobrevin. However, the secretory machinery is probably more complex than previously thought, as other "plasma membrane" proteins of the SNARE complex, such as syntaxin (Tagaya *et al.*, 1995) and SNAP-25 (Tagaya *et al.*, 1996), have now been shown to localize to the membrane of the secretory granules themselves. It would be interesting to evaluate if these proteins, including the N-type VOCC, are directly inserted in the membrane of the newly formed secretory granules, or if they reach the granules utilizing an endosome-mediated recycling pathway, after they have been constitutively inserted in the plasma membrane.

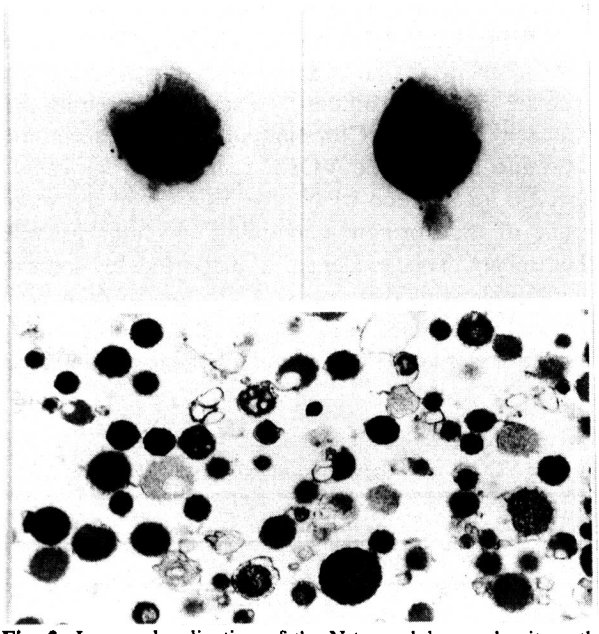

Fig. 2. Immunolocalization of the N-type alpha$_{1B}$ subunit on the membrane of adrenal chromaffin granules. Immonolabeling was performed on sections of purified secretory granules with the aid of the same alpha$_{1B}$ specific antibodies used for western blotting (CW14), followed by anti rabbit-colloidal gold secondary antibodies.

Summarizing, most of our evidence suggests a specific localization of at least some of the intracellular N-type VOCCs in the membrane of the secretory granules. However, some of our data also suggest some caution in this respect. In PC12 cells, for example, we did not find a significant co-localization of alpha$_{1B}$ immunoreactivity with Chromogranin B immunoreactivity in double-labeling immunofluorescence experiments (Sher *et al.*, unpublished results). We are not sure if this latter evidence points to the presence of N-type VOCCs in only a "subpopulation" of the heterogeneous secretory granules of PC12 cells, or to the presence of N-type VOCCs in a still uncharacterized "third type" of secretory vesicle (not granules nor microvesicles), or even in a recycling endosomal compartment.

Although the evidence for the presence of *voltage-operated* calcium channels in the membrane of secretory organelles is novel, an important precedent is the demonstration of the presence of *calcium release channels* in the same organelles, as recently reported (Blondel *et al.*, 1995; Petersen, 1996 and references therein). These *calcium release channels* are supposed to participate in the amplification of calcium signals near release sites. Whether the N-type VOCCs also have a similar "intracellular" role, or whether they are only "in transit" to the plasma membrane, is a major question to be addressed in the future.

EVIDENCE FROM OTHER SYSTEMS

The regulation of the expression of L-type VOCCs has been relatively throughly studied over the years. In particular, it was shown that L-type VOCC "agonists" and "antagonists" cause the "down-regulation" and "up-regulation," respectively, of L-type VOCCs, in a similar way to more classical receptor agonists and antagonists (Ferrante and Triggle, 1990). Also in the case of voltage-dependent sodium channels, agonist and antagonist toxins have been shown

to cause sodium channel "down-regulation" and "up-regulation," respectively (Sherman and Catterall, 1984; Bar-Sagi and Prives, 1985; Dargent and Couraud, 1990). Both these examples are in line with our data showing a calcium antagonist-induced increase in surface N-type VOCCs due to the recruitment of intracellular channels.

Intracellular pools of both neuronal nicotinic receptors (Stollberg and Berg, 1987) and sodium channels (Schmidt *et al.*, 1985) have been described. A translocation of sodium channels to the plasma membrane has also been demonstrated in isolated neuronal growth cones (Wood *et al.*, 1992).

More divergent from ours are some results reported in the literature on the effects of short- and long-term depolarizations on the expression of L-type VOCCs. In these studies, depolarization was found to *decrease* the surface expression of L-type VOCCs and, in some cases, this was because of channel internalization (Franklin *et al.*, 1992; Liu *et al.*, 1994; Feron and Godfraind, 1995). As described above, we found depolarization to *increase* the surface expression of N-type VOCCs. Experiments are ongoing in order to define if this difference represents a real *differential regulation* of VOCC subtypes in response to the same stimuli, or to specific cellular or experimental differences. Further support of our findings was reported by Garcia *et al.* (1994) who found that in rat hippocampal neurons *in vitro*, transient and repetitive depolarizations with high KCl resulted in an increased expression of high - threshold calcium channels.

Finally, the neuroendocrine bag cell of Aplysia is a very well-characterized system where a process similar to our "secretagogue-induced" VOCC recruitment seems to operate. In these cells TPA stimulates, in a few minutes, the "recruitment" to the cell surface of new VOCCs which have different biophysical properties with respect to the preexisting ones (Strong *et al.*, 1987). Furthermore, the appearance of new "calcium influx sites" in Aplysia growth cones is paralleled by an increase in growth-cone surface area, suggesting that the new VOCCs were incorporated by exocytosis (Knox *et al.*, 1992). Finally, an intracellular, vesicular, localization of the calcium channels to be recruited in Aplysia bag cells was recently demonstrated by the same authors (White *et al.*, 1997). Interestingly, the immunolocalization results in Aplysia suggested that the intracellular VOCCs are present in vesicles which were not the hormone-containing granules but another, still undefined, vesicle type (White *et al.* 1997).

PERSPECTIVES

An important task is now to correlate the short- and long-term regulation of the different types of VOCCs and to study whether there are any significant physiological differences. As pointed out before, it seems that there might be some differences in how L-type and N-type VOCCs are regulated by depolarization. More interesting would be to compare the regulation of N-type and P/Q-type VOCCs, since these two VOCC types, more than the L-type, share a relevant role in the control of secretion, as well as specific biochemical interactions in the nerve terminals (Bezprozvanny *et al.*, 1995). Besides comparing the regulation of the different VOCC subtypes, it would be even more interesting to evaluate if, in the same cell, different isoforms of the N-type VOCC could be differentially regulated. Further work, possibly conducted in the same experimental systems, is required to obtain a more definitive picture of these phenomena.

The "up" and "down" regulation of the different VOCC subtypes could be important for the physiological regulation of cell activity and, in particular, could underlie specific forms of synaptic plasticity that, given the variable localization of the different VOCC subtypes, could express themselves both pre- or postsynaptically. Furthermore, VOCC recruitment could also participate in one or more of the several forms of calcium channel "facilitation" described in neurosecretory cells (Dolphin, 1996).

On the other hand, the regulated expression of surface VOCCs might also have pathological and therapeutic implications: in acutely or chronically depolarized cells, such as in neurons of epileptic foci or ischemic areas, a change in the surface expression of different VOCC subtypes, possibly secondary to secretion-induced recruitment, could cause a pathological imbalance in calcium influx and homeostasis. Knowing how VOCC expression/activity changes during these events could help both in elucidating the pathogenesis of cell damage and death, and also in defining new therapeutic targets.

Finally, there is an obvious interest in the long-term regulation of VOCC expression in the course of chronic treatments with calcium antagonists, in order to plan therapeutic regimes aimed at avoiding tolerance and/or withdrawal effects.

In summary, the regulation of VOCC expression in neurosecretory cells is an exciting, growing field of research where new aspects of basic cellular neurobiol-

ogy will likely show up having important physiological and therapeutic implications.

ACKNOWLEDGMENTS

We thank Dr. John R. Boot for critically reviewing the manuscript. The financial support of Telethon Italy (grant 858 to E.S.) to some of the studies here reported is greatly acknowledged. A.C. is supported by an AIRC fellowship.

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