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ORIGINAL ARTICLE

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Coupling of L-type calcium channels to neurotransmitter release at mouse motor nerve terminals

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Abstract Previously, we have presented evidence for the presence of L-type voltage-dependent Ca^{2+} channels (VDCC) in 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, (acetoxymethyl)ester (BAPTA-AM)-incubated motor nerve terminals (MNTs) of the levator auris muscle of mature mice. The aim of the present work was to study the coupling of these L-type VDCC to neurotransmitter release by inhibiting protein phosphatases. We thus studied the effects of the protein phosphatase inhibitors okadaic acid (OA) and pervanadate on quantal content (QC) of transmitter release with the P/Q-type channels fully blocked. The QC was not significantly different under the three experimental conditions tested: incubation with dimethylsulphoxide (DMSO), ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, (acetoxymethyl)ester (EGTA-AM) and BAPTA-AM. After preincubation with OA (1 μM), but not with pervanadate, QC increased substantially in the BAPTA-AM-incubated (up to 400%) MNT, but not in those incubated with DMSO or EGTA-AM. The OA-induced increment of QC was attenuated greatly (~95% reduction) by preincubation with either nitrendipine (10 μM) or calciseptine (300 nM). The effect of OA (1 μM) and pervanadate (0.1 mM) on spontaneous neurotransmitter release was also studied. After preincubation with OA, but not pervanadate, miniature end-plate potential (MEPP) frequency increased only in the BAPTA-AM-incubated MNT (up to 700% increment). This response was attenuated (by ~80%) by nitrendipine (10 μM) or calciseptine (300nM). In contrast, neither ω-agatoxin IVA (120 nM) nor ω-conotoxin GVIA (1 μM) affected this OA-induced increment significantly. We also evaluated the relationship between QC and extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$) in BAPTA-AM-incubated MNT. Under conditions in which only P/Q-type VDCC were available to participate in neurotransmitter release, QC increased as $[Ca^{2+}]_o$ was raised from 0.5 to 2 mM. However, when only L-type VDCC were available, QC increased when $[Ca^{2+}]_o$ increased from 0.5 to 1 mM, but decreased significantly at 2 mM. The mean latency for P/Q-type VDCC-mediated EPP was 1.7–1.9 ms; for L-type VDCC-mediated EPP, 1.9–2.5 ms. The rise time of the L-type VDCC mediated EPP was significantly slower than that mediated by P/Q-type VDCC. Preincubation with H-7 (100 μM), a potent inhibitor of protein kinase C (PKC) and adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase (PKA), attenuated the OA-induced increment of both QC and MEPP frequency (50% and 70% decrement, respectively), suggesting the participation of at least these two protein kinases in the coupling of L-type VDCC. In summary, our results show coupling of L-type VDCC to neurotransmitter release when protein phosphatases are inhibited and intracellular $[Ca^{2+}]$ is buffered by the fast chelator BAPTA.

Keywords Calcium channel · Motor nerve terminal · Cell-permeant calcium buffers · BAPTA-AM · EGTA-AM · Serine/threonine phosphorylation · Okadaic acid

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Introduction

Voltage dependent calcium channels (VDCC) play a key role in neuronal signalling as mediators of Ca^{2+} entry during the process of neurotransmitter release [26, 30]. At the mature mammalian motor nerve terminal (MNT), neurotransmitter release is mediated by VDCC of the P/Q- but not L- or N-types [47]. During development, however, changes in the VDCC types coupled to neurotransmitter release have been described in avian [20], amphibian [19] and mammalian MNT [38]. Besides, at the mouse neuromuscular junction, the presence of new types of VDCC mediating neurotransmitter release has also been reported during processes such as re-innerva-

tion [27], and functional recovery from *botulinum* toxin type-A poisoning [39].

During neurotransmitter release, intracellular Ca^{2+} is arranged in "local microdomains" of elevated concentration that originate from VDCC [29]. Ca^{2+}-triggered cellular events behave according to the localisation of the Ca^{2+}-sensitive element with respect to these microdomains. This means that intracellular Ca^{2+} constitutes a complex signal that can be shaped by factors that alter Ca^{2+} buffering and/or Ca^{2+} diffusion. Specifically, studies using intracellular Ca^{2+} chelators provide additional evidence in this respect and are effective tools for disrupting specific interactions between channels and synaptic proteins [33]. Cell-permeant Ca^{2+} chelators, which become active intracellular Ca^{2+} buffers after the action of cytoplasmic esterases, have been accepted as a non-invasive means of reducing the elevation of intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$), despite the fact that their final intracellular concentration is unknown [44, 46]. 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) and ethyleneglycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), the most widely used Ca^{2+} chelators, have similar affinity constants (K_d) for Ca^{2+}, but EGTA has a binding rate constant (k_{on}) for Ca^{2+} more than 100 times slower [2].

L-type VDCC are thought to play an important role in neurotransmitter release [3, 5, 15, 27, 39]. Interestingly, the activity of L-type VDCC is controlled by voltage as well as by Ca^{2+}-dependent inactivation, the latter being mediated by the fast Ca^{2+} influx through the channel [32, 41]. The local microdomains of elevated $[Ca^{2+}]$ generated by the influx of Ca^{2+} through the pore of the channel account for L-type VDCC Ca^{2+}-dependent inactivation. We have shown previously [48] that cell-permeant Ca^{2+} chelators, such as the (acetoxymethyl)ester of BAPTA (BAPTA-AM), can unmask L-type perineural Ca^{2+} currents. This allowed us to postulate that BAPTA acts by lowering the $[Ca^{2+}]$ quickly enough so as to decrease the $[Ca^{2+}]$ sensed by the L-type VDCC "inactivation machinery". Therefore, when using BAPTA-AM, the level of the Ca^{2+} signal at the local microdomains is reduced, thus preventing inactivation. This revealed an L-type VDCC component in the perineural Ca^{2+} currents in the mature mammalian neuromuscular junction. Contrary to findings in the squid giant presynaptic terminal [2], the final intracellular concentration of BAPTA in our experiments in mouse MNT (i.e. normal saline solution and low rate of stimulation) did not affect the quantal content (QC) of transmitter [48]. This indicates that, in mature mammalian MNT, the coupling between P/Q-type VDCC and neurotransmitter release is so tight that it cannot be disrupted, even by the action of a fast Ca^{2+} chelator. Experiments using fura-2 as an indicator suggest that the $[Ca^{2+}]_i$ transient amplitude is less attenuated by EGTA-AM than by BAPTA-AM [46]. Similarly, we detected a nitrendipine-sensitive, L-type presynaptic perineural Ca^{2+} current in MNT incubated with BAPTA-AM, but not with EGTA-AM. This L-type component was not, however, implicated directly in nerve-evoked neurotransmitter release [48]. Thus, our previous results have suggested the existence of Ca^{2+}-inactivated L-type VDCC in mature MNT, which can be unmasked by the action of BAPTA. Interestingly, Arenson and Gill [4] have described the coupling of "silent" (not directly involved in neurotransmitter release) L-type VDCC to neurotransmitter release at frog MNT after the inhibition of serine/threonine phosphatases with okadaic acid (OA).

Protein phosphorylation is a relatively fast process that plays a cardinal role in neuronal excitability regulation, plasticity and excitotoxicity. The balance between protein kinases and protein phosphatases regulates phosphorylation. In fact, when protein phosphatases are inhibited, numerous cell proteins are trapped in their phosphorylated forms. Most of the protein phosphatases described can be classified as either protein serine/threonine phosphatases or protein tyrosine phosphatases [25, 43, 45]. In particular, the function of L-type VDCC is regulated by either serine/threonine [3, 5, 8, 15, 18, 21, 41, 42] or tyrosine [16, 34] phosphorylation. Furthermore, many proteins involved in neurotransmitter release are known to be regulated by phosphorylation [43, 45].

The aim of the present work was to examine the possibility that, when intracellular Ca^{2+} is buffered by the fast Ca^{2+} chelator BAPTA, L-type VDCC couple to neurotransmitter release when protein phosphatases are inhibited. Some of these data have been reported in abstract form [12].

Materials and methods

Experiments were carried out on the left levator auris longus muscle of male Swiss mice weighing 20–30 g. The animals were cared for in accordance with national guidelines for the humane treatment of laboratory animals, which are comparable to those of the US National Institutes of Health. Animals were anaesthetised with an overdose of 2% tribromoethanol (0.15 ml/10 g body wt) and exsanguinated immediately. The corresponding muscle with its nerve supply was excised and dissected on a Sylgard-coated Petri dish containing physiological saline (normal saline solution) of the following composition (mM): NaCl 137, KCl 5, $CaCl_2$ 2, $MgSO_4$ 1, $NaHCO_3$ 12, Na_2HPO_4 1 and glucose 11; continuously bubbled with 95% O_2/5% CO_2; pH 7.2–7.4. The preparation was then transferred to a recording chamber of 1.0 ml. Experiments were performed at room temperature (20–23 °C).

Intracellular recordings

Evoked endplate potentials (EPP) were recorded intracellularly with conventional glass microelectrodes filled with 3 M KCl (10–15 MΩ resistance). Records were rejected if the 10–90% EPP rise time exceeded 1 ms. The resting membrane potential of the muscle fibres (V_m) ranged from –60 to –80 mV and neither OA or H-7 affected it. After impaling a muscle fibre, the nerve was continuously stimulated at 0.5 Hz via two platinum electrodes coupled to a pulse generator and a stimulus isolation unit.

In dimethylsulphoxide- (DMSO, the chelator vehicle), EGTA-AM- and BAPTA-AM-incubated MNT, in the presence of either ω-agatoxin IVA or ω-agatoxin TK (ω-Aga, 120 nM), failure of neuromuscular transmission, i.e. the absence of EPP during nerve stimulation was observed frequently. In fact, 120 nM ω-Aga strongly blocks (up to 95%) the P/Q-type VDCC present in this

muscle [28]. Hence, the meanQC was estimated by the failures method: $QC=\ln(N/n_0)$ where N is the total number of episodes at 0.5 Hz (at least 100) and n_0 is the number of trials in which the response failed [24].

QC was calculated in the presence of 120 nM ω-Aga using a saline solution modified to prevent the Ca^{2+}-dependent inactivation of L-type VDCC (see below) and containing (mM): NaCl 137, KCl 5, $CaCl_2$ 1, $MgSO_4$ 1, $NaHCO_3$ 12, Na_2HPO_4 1 and glucose 11; continuously bubbled with 95% O_2/5% CO_2, pH 7.2–7.4. In addition, other bath $[Ca^{2+}]$ ($[Ca^{2+}]_o$: 0.5 and 2 mM) were used at the same $[Mg^{2+}]$ to evaluate the relationship between QC and $[Ca^{2+}]_o$.

The relationship between QC and $[Ca^{2+}]_o$ was calculated also under conditions in which only P/Q-type (i.e. exposure to 40 nM ω-Aga and 300 nM calciseptine to block L-type VDCC fully, whilst blocking the P/Q-type VDCC only partially) or only L-type (i.e. fully blocking the P/Q-type VDCC by 120 nM ω-Aga) VDCC were available to participate in neurotransmitter release (see [28] for the ω-Aga concentration/response relationship in this muscle). Under both conditions, EPP latency (computed as the time elapsed from the stimulation artefact to the foot of the EPP) was also analysed when the 10–90% EPP rise time was 1 ms or less (see Fig. 3B). OA concentration was kept constant at 1 µM.

Spontaneous miniature EPPs (MEPP) were recorded intracellularly with conventional glass microelectrodes filled with 3 M KCl (10–15 MΩ resistance) in normal saline solution for periods of 1–2 min and stored on disk for further computer analysis.

Recording electrodes were connected to an amplifier (Axoclamp 2A, Axon Instruments, Foster City, Calif., USA). A distant Ag-AgCl electrode connected to the bath solution via an agar bridge (agar 3.5% in 137 mM NaCl) was used as reference. The signals were digitised (Digidata 1200; Axon Instruments), acquired, stored and analysed using appropriate software (PClamp v. 6.0 and Axoscope v. 1.0, Axon Instruments). Origin 3.0 software (Microcal Software, Northampton, Mass., USA) was used to plot the data.

Loading procedure

MNTs were loaded with BAPTA or EGTA as previously described [48]. In brief, the nerve-muscle preparation was incubated with 10 µM of the AM form of the Ca^{2+} chelator for 2 h at 37 °C in Ca^{2+}-free saline solution. The cell-permeant Ca^{2+} chelators BAPTA-AM and EGTA-AM have similar structures and molecular weights and were used at equimolar concentrations; their accumulation in MNT should therefore be of the same order of magnitude. After incubation with chelators, the preparations were then washed for 15 min with the Ca^{2+}-free solution and finally for 15 min with normal saline solution.

Toxins and chemicals

Tribromoethanol was purchased from Aldrich. Nitrendipine, OA and H-7 dihydrochloride were purchased from RBI (Natick, Mass., USA). Bovine serum albumin (BSA), and all other salts were of analytical grade and purchased from Sigma (St. Louis, Mo., USA). BAPTA-AM and EGTA-AM were purchased from Molecular Probes (Eugene, Ore., USA). Orthovanadate was kindly provided by Dr. Rodolfo R. Llinás (New York University Medical Center, USA). The synthetic polypeptides ω-conotoxin GVIA (ω-CgTX) and calciseptine were purchased from Alomone (Jerusalem, Israel). To block P/Q-type VDCC we used either ω-agatoxin IVA (Peptide Institute; Tokyo, Japan) or ω-agatoxin TK (Alomone). In these experiments, both control and toxin-treated fibres were assayed in the presence of a final concentration of 0.01% BSA.

Nitrendipine was made up as a 50 mM stock solution in DMSO, stored at 4 °C and protected from light. Experiments in the presence of nitrendipine were carried out in the absence of direct illumination. BAPTA-AM and EGTA-AM were prepared as 10 mM stock solutions in DMSO and stored at –20 °C. Aliquots were diluted in the Ca^{2+}-free saline solution to yield the final concentration of 10 µM. The serine/threonine phosphatase inhibitor OA was made up as 10 mM stock solution in DMSO and stored at –20 °C. Aliquots were diluted in the saline solution in use to yield the final concentration of 1 µM. Experiments were performed from at least 20 min after OA application. A preincubation of 60 min with either nitrendipine or toxins was allowed before application of OA. The final DMSO concentration in control and drug-treated preparations was 0.1% (v/v). The parameters under study were not affected by either the incubation period or DMSO (two muscles, data not shown).

The tyrosine phosphatase inhibitor pervanadate was prepared as previously described [31]. In brief, a stock solution was prepared by mixing 1 vol 50 mM H_2O_2 with 5 vol 10 mM sodium orthovanadate in Ca^{2+}-free saline solution and then incubating at room temperature for 10 min. This stock solution was diluted 1:10 in normal saline solution to a final concentration of 0.1 mM vanadate and 0.1 mM H_2O_2. The inhibitor of adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase (PKA) and protein kinase C (PKC), H-7 dihydrochloride, was made up as a 10 mM stock solution in distilled water and stored at –20 °C. Aliquots were diluted in the saline solution in use to yield the final concentration of 100 µM. Preparations were preincubated in H-7 for 60 min before the application of OA.

Data presentation, fitting and statistics

In figures, changes in both spontaneous MEPP and QC are presented as fractions of the control values. The fraction of control was calculated as the mean of the ratio: test MNT/mean of control MNT. Under our conditions, the relation between QC and $[Ca^{2+}]_o$ was a power relationship: $QC=b\cdot([Ca^{2+}]_o)^m$. This can be linearized as a double-logarithmic plot: $\log(QC)=m\cdot\log([Ca^{2+}]_o)+\log b$ where m and $\log b$ are the slope and the intercept with the abscissa, respectively. The significance of differences between means was evaluated by the Welch's t-test (two-tailed, for unpaired values and not assuming equal variances). Histograms of P/Q- and L-type VDCC mediated EPP latency were fitted to a single normal distribution ($r^2>0.9$). The significance of differences in EPP latency and 10–90% rise times were evaluated by one-way ANOVA. $P<0.05$ was regarded as significant.

Results

Effect of OA and pervanadate on QC in DMSO-, BAPTA-AM- and EGTA-AM-incubated MNT

Evoked neurotransmitter release mediated by L-type VDCC was investigated after preincubation with 120 nM ω-Aga, to block the P/Q-type VDCC in these MNT [28]. Under these conditions, we studied the effects of okadaic acid (OA) and pervanadate, specific serine/threonine and tyrosine protein phosphatase inhibitors, respectively.

In the presence of 120 nM ω-Aga, frequent EPP absences (i.e. failures) occurred after nerve stimulation in the DMSO- (control), EGTA-AM- and BAPTA-AM-incubated MNT (see Fig. 1A, B and C left, respectively). Thus, QC of DMSO-, EGTA-AM- and BAPTA-AM-incubated MNT were not significantly different (DMSO 0.16±0.03, n=20 MNT, 2 muscles; BAPTA-AM, 0.11±0.02, n=20 MNT, 2 muscles; EGTA-AM 0.13±0.04, n=55 MNT, 3 muscles).

Adding OA to the bath solution decreased the number of transmission failures significantly in BAPTA-AM-in-

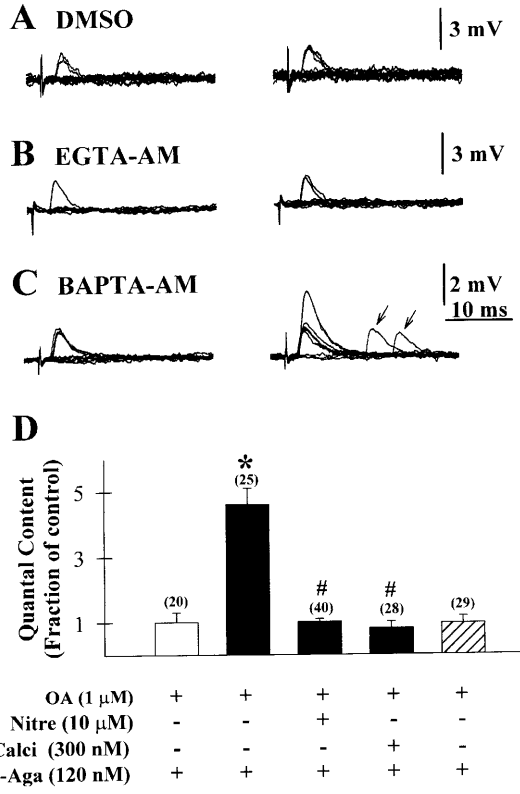

Fig. 1A–D Effect of okadaic acid (*OA*) on quantal content (QC) in dimethylsulphoxide (*DMSO*)-, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid, (acetoxymethyl)ester (*BAPTA-AM*)- and ethyleneglycol-bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (acetoxymethyl)ester (*EGTA-AM*)-incubated motor nerve terminals (MNT) in the levator auris muscle of mature mice. **A**, **B**, Superimposed consecutive traces during nerve stimulation before (*left*) and 30 min after (*right*) bath application of 1 μM OA recorded in the same DMSO- and EGTA-AM-incubated MNT, respectively. Resting membrane potential (V_m) was –70 and –50 mV, respectively. **C** As in **A** and **B** but in BAPTA-AM-incubated MNT. V_m –75 mV. *Arrows* indicate spontaneous miniature endplate potentials (MEPP). **D** Summary of the effect of OA on quantal content calculated by the failures method (see Materials and methods) in DMSO- (*open bars*), BAPTA-AM- (*filled bars*) and EGTA-AM-incubated (*hatched bars*) MNT in the presence of 120 nM ω-agatoxin IVA or ω-agatoxin TK (ω-Aga). In all conditions, data were obtained after 60 min preincubation with either nitrendipine (*Nitre*) or calciseptine (*Calci*) and 30 min after addition of OA. In all cases, stimulation frequency was 0.5 Hz. Stimulation artefacts were reduced for clarity. The numbers of MNT studied in each case are given in *parentheses* (at least two muscles per *bar*). Data are presented as means±SEM of the ratio: test MNT/mean of control MNT. **P*<0.001 vs. BAPTA-AM-incubated group before OA addition; #*P*<0.001 vs. BAPTA-AM-incubated group in the presence of OA

cubated MNT (Fig. 1C, right), but not in MNT incubated with DMSO or EGTA-AM (Fig. 1A and B, right, respectively). After preincubation with OA (1 μM), the QC of BAPTA-AM-incubated MNT increased drastically (Fig. 1D, filled bars, 0.46±0.05, *n*=25 MNT, 3 muscles, *P*<0.001) but not that in DMSO- (Fig. 1D, open bar, 0.18±0.05, *n*=12 MNT, 2 muscles; *P*>0.05) or EGTA-AM-incubated (Fig. 1D, hatched bar, 0.12±0.03, *n*=29 MNT, 3 muscles; *P*>0.05) MNT. The effects induced by

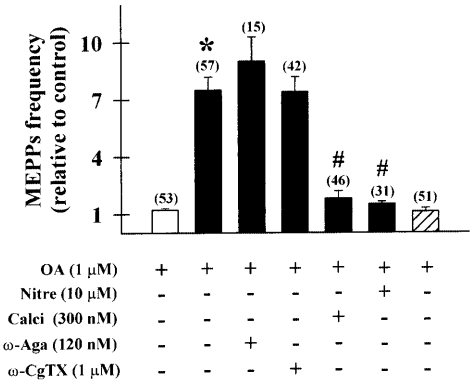

Fig. 2 Effect of OA on spontaneous MEPP frequency in DMSO- (*open bar*), BAPTA-AM- (*filled bars*) and EGTA-AM- (hatched bar) incubated MNT. Spontaneous MEPP were recorded in normal saline solution. The number of MNT studied in each case is given in *parentheses* (at least two muscles per bar). In all conditions, data were obtained after 60 min preincubation with ω-Aga, ω-conotoxin GVIA (*ω-CgTX*), nitrendipine or calciseptine and 30 min after addition of OA. Data are presented as means±SEM of the ratios: test MNT/mean of control MNT. **P*<0.001 vs. BAPTA-AM-incubated group before OA addition; #*P*<0.001 vs. BAPTA-AM-incubated group in the presence of OA

OA were reversed after 15 min washout (three muscles, data not shown).

Preincubation of BAPTA-AM-treated MNT with the L-type VDCC blockers nitrendipine (10 μM) or calciseptine (300 nM) drastically reduced the QC increment induced by OA (Fig. 1D; filled bars, nitrendipine: 0.11±0.02, *n*=40 MNT, 2 muscles; calciseptine: 0.08±0.03, *n*=28 MNT, 2 muscles; *P*<0.001). This effect was also reversible (two muscles, data not shown). There was no significant difference between these L-type blockers.

We also studied the effect of pervanadate (0.1 mM), a specific tyrosine phosphatase inhibitor, on the coupling of L-type VDCC to neurotransmitter release in BAPTA-AM-incubated MNT. After 30 min preincubation with pervanadate, QC was not significantly different (fraction of control: 0.78±0.16, *n*=59 MNT, 3 muscles, data not shown). These results indicate OA-induced coupling of L-type VDCC to evoked neurotransmitter release in BAPTA-AM-incubated MNT.

Effect of OA and pervanadate on spontaneous MEPP frequency in DMSO-, BAPTA-AM- and EGTA-AM-incubated MNT

Basal MEPP frequency was not significantly different between DMSO-, EGTA-AM or BAPTA-AM-incubated MNT (49±4.8, 43.5±7.8 and 42.5±6.9 min^{-1}, respectively). After preincubation with 1 μM OA, however, MEPP frequency increased up to 7 times in BAPTA-AM-incubated MNT (313.3±29 min^{-1}, *n*=57 MNT, 4 muscles; *P*<0.001). In contrast, OA elicited no increment in MEPP frequency in MNT incubated with DMSO (70±7 min^{-1}, *n*=53 MNT, 3 muscles) or EGTA-AM (73±12 min^{-1}, *n*=34 MNT, 3 muscles) (Fig. 2). More-

over, the OA-induced increment in BAPTA-AM-incubated MNT was prevented by nitrendipine (10 μM, 63.8±6.2 min^{-1}, *n*=31 MNT, 2 muscles, *P*<0.001) or calciseptine (300 nM, 73.6±17 min^{-1}, *n*=46 MNT, 4 muscles, *P*<0.05). Neither preincubation with ω-Aga (120 nM, 382±30 min^{-1}, *n*=15 MNT, 2 muscles,) nor ω-CgTX (1 μM, 307.5±32, *n*=42 MNT, 2 muscles) affected the OA-induced increment in MEPP frequency. These results thus suggest an OA-induced coupling of L-type VDCC with spontaneous neurotransmitter release in BAPTA-AM-incubated MNT. After 30 min preincubation with pervanadate, spontaneous MEPP frequency in BAPTA-AM-incubated MNT remained unchanged (fraction of control: 0.88±0.11, *n*=68 MNT, 3 muscles; data not shown), suggesting that pervanadate did not affect the coupling status of L-type VDCC with spontaneous neurotransmitter release in BAPTA-AM-incubated MNT.

Parameters of P/Q- and L-type VDCC-mediated neurotransmitter release in BAPTA-AM-incubated MNT

We investigated the relationship between QC and $[Ca^{2+}]_o$ (see Materials and methods) to characterise further the coupling between L-type VDCC and evoked neurotransmitter release. QC was calculated under conditions in which only P/Q-type VDCC were available to participate in the process of release in BAPTA-AM-incubated MNT (i.e. exposure to 40 nM ω-Aga and 300 nM calciseptine). Under these conditions, QC increased over the whole range of $[Ca^{2+}]_o$ employed (Fig. 3A; filled circles). The straight line fitted to the points had a slope of 0.7 (Fig. 3A; continuous line; r^2>0.99). On the other hand, under conditions under which only L-type VDCC were available (i.e. exposure to 120 nM ω-Aga), QC increased on raising $[Ca^{2+}]_o$ from 0.5 to 1 mM but decreased significantly at 2 mM (Fig. 3A; open circles; *P*<0.05). The reduction in QC caused by 2 mM $[Ca^{2+}]_o$ suggests that L-type VDCC might be inactivated by this $[Ca^{2+}]_o$ (see Discussion). The straight line fitted to the two first points had a slope of 1.4 (Fig. 3A; dotted line; r^2>0.99).

Next, we studied the latency and rise time of EPP during neurotransmitter release mediated by either P/Q- or L-type VDCC in BAPTA-AM-incubated MNT. Figure 3B shows representative EPP when only P/Q-type (Fig. 3B, *i*) or L-type (Fig. 3B, *ii*) VDCC were available to participate in neurotransmitter release. As shown in Fig. 3C, the histograms of EPP latency values corresponding to both conditions were fitted by a single normal distribution (continuous lines; r^2>0.9). The mean latency for P/Q-type VDCC-mediated EPP was 1.91±0.01 ms; for L-type VDCC-mediated EPP, 2.35±0.04 ms (*P*<0.001 vs. P/Q-type VDCC-mediated EPP latency). Differences in the EPP rise time were also observed. P/Q-type VDCC-mediated EPP were faster than those mediated by L-type VDCC (0.65±0.03 ms, *n*=69 and 0.83±0.02, *n*=73, for P/Q- and L-type VDCC-mediated EPP, respectively; *P*<0.001).

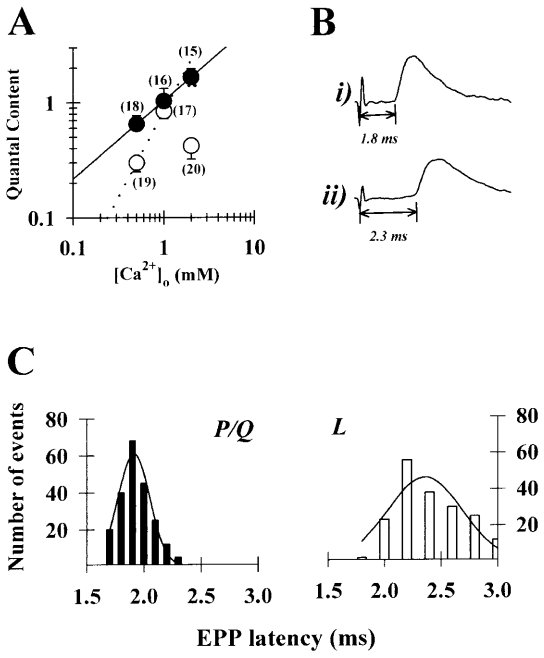

Fig. 3A–C Parameters of P/Q- and L-type voltage-dependent Ca^{2+} channel (VDCC)-mediated neurotransmitter release in BAPTA-AM-incubated MNT. **A** Double-logarithm plot of QC as a function of extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$). QC was calculated by the failures method (see Materials and methods) under conditions in which only P/Q-type VDCC (●, presence of 40 nM ω-Aga and 300 nM calciseptine), or only L-type VDCC (○, presence of 120 nM ω-Aga) were available to participate in neurotransmitter release. In both cases, OA concentration was constant (1 μM) whilst $[Ca^{2+}]_o$ was 0.5, 1 or 2 mM. $[Mg^{2+}]$ was constant. The number of MNT studied in each case is shown in *parentheses* (at least two muscles per point). Data presented as means±SEM for P/Q-type (*continuous line*; r^2>0.99) and L-type (*dotted line*; r^2>0.99) VDCC were fitted to the linear equation: Log(QC)= $m \cdot \log[Ca^{2+}]_o + \log b$ where *m* and log*b* are the slope and *abscissa* intercept, respectively. **P*<0.05 vs. L-type VDCC at $[Ca^{2+}]_o$= 1 mM. **B** Representative EPP recorded during nerve stimulation under conditions in which only P/Q-type (*i*) or L-type (*ii*) VDCC were available to participate in neurotransmitter release (see Materials and methods). **C** EPP latency distribution histograms obtained under conditions in which only P/Q- (■) or L- (□) -type VDCC were available to participate in neurotransmitter release. Histograms comprised 203 and 230 EPP, respectively. The *continuous lines* show the fit to a single normal distribution (r^2>0.9)

These results indicate that neurotransmitter release mediated by these two types of VDCC in BAPTA-AM-incubated MNT have a different Ca^{2+}-co-operativity. The differences observed in EPP latency and rise time may result from the spatial relationship between the VDCC and the active zone and/or different kinetics of activation of these channels.

Role of protein kinases in the OA-induced coupling of L-type VDCC to neurotransmitter release in BAPTA-AM-incubated MNT

To determine whether phosphorylation or some other mechanism is responsible for the OA-induced effects de-

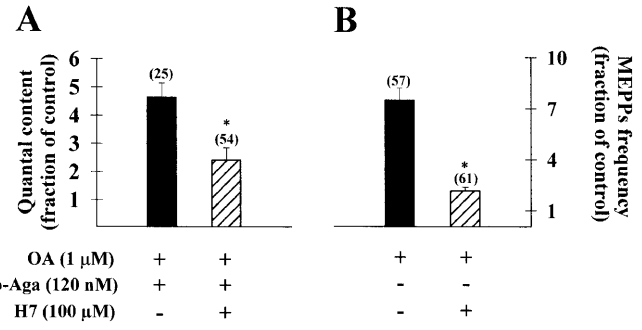

Fig. 4A, B Role of protein kinases in the OA-induced coupling of L-type VDCC to neurotransmitter release in BAPTA-AM-incubated MNT. **A** Effect of OA on QC in the absence (■) or presence (□) of H-7 (100 μM) in BAPTA-AM-incubated MNT. QC was calculated by the failures method in the presence of 120 nM ω-Aga. **B** As for **A**, but for MEPP frequency. The preparations were preincubated with H-7 for 60 min before the application of OA. The number of MNT studied in each case is shown in *parentheses* (at least two muscles per *bar*). Data are presented as means±SEM of the ratio: test MNT/mean of control MNT. **P*<0.001 vs. MNT treated with BAPTA-AM and incubated with OA without H-7

scribed above, we evaluated whether H-7, a potent inhibitor of both PKA and PKC [22], was able to affect the OA-induced increment in QC and MEPP frequency. Figure 4 shows that preincubation of the BAPTA-AM treated MNT with H-7 (100 μM), attenuated the OA-induced increment of both QC (Fig. 4A, 0.26±0.05, n=54 MNT, 3 muscles, P<0.001) and MEPP frequency (Fig. 4B, 91.5±9.4 min^{-1}, n=61 MNT, 3 muscles, P<0.001). Preincubation with 100 μM H-7 alone did not affect either QC or MEPP frequency (two muscles; data not shown).

These results are consistent with the hypothesis that OA exerted its effects by enhancing the serine/threonine phosphorylation state of cellular proteins and suggests that at least PKA and/or PKC might be involved in the coupling of L-type VDCC to neurotransmitter release. Other types of PKs may also be involved.

Discussion

The presence of a nitrendipine-sensitive, L-type presynaptic perineural Ca^{2+} current in BAPTA-AM-, but not in DMSO- or EGTA-AM-incubated MNT of the levator auris muscle of mature mice has been described recently. These results led us to postulate that fast Ca^{2+} chelators, such as BAPTA, unmask the L-type Ca^{2+} currents by eliminating Ca^{2+} channel inactivation. This L-type component was not, however, directly implicated in nerve-evoked neurotransmitter release and the coupling of P/Q-type VDCC with neurotransmitter release was not disrupted by BAPTA [48].

Here we studied the effect of OA and pervanadate, specific serine/threonine and tyrosine protein phosphatase inhibitors, respectively, on evoked and spontaneous neurotransmitter release in BAPTA-AM- and EGTA-AM-incubated MNT. It is well known that Ca^{2+} entering the terminal through P/Q-type VDCC mediates nerve-evoked, but not spontaneous, transmitter release in these MNT [28, 47]. Considering this, and seeking to detect any effect involving L-type VDCC, we studied QC in the presence of a high concentration of ω-Aga, under which conditions changes in QC following the action of OA or pervanadate cannot be accounted for by an effect of these drugs on P/Q-type VDCC.

Under these conditions, QC remained very low and no significant differences were observed when MNT were loaded with fast or slow cell-permeant Ca^{2+} chelators. Interestingly, QC remained very low after loading the MNT with the fast chelator BAPTA even though, presumably, under these conditions Ca^{2+} inactivation is removed and Ca^{2+} influx through L-type VDCC is maintained for a longer period. However, in the presence of OA (1 μM) there was a significant and reversible increment in both QC and spontaneous MEPP frequency in BAPTA-AM-incubated MNT, but not in those incubated with DMSO or EGTA-AM. This suggests that the speed, and not the capacity, of the Ca^{2+} chelator was relevant for this process.

It is well known that OA at this concentration inhibits the serine/threonine phosphatases PP1, PP2A and PP4 [25]. Likewise, effects of OA have been described previously on MNT [1, 4, 6] and chromaffin cells [5]. In particular, Arenson and Gill [4] have shown the coupling of L-type VDCC to neurotransmitter release in the frog MNT as a consequence of the action of OA.

Under our conditions, the OA-induced increment of both QC and spontaneous MEPP frequency at BAPTA-AM-incubated MNT was blocked by nitrendipine (10 μM) or calciseptine (300 nM), indicating the participation of L-type VDCC in this process. The regulation of the function of L-type VDCC by serine/threonine phosphorylation has been described extensively [3, 5, 8, 15, 18, 21, 41, 42]. Finally, although the function of L-type VDCC is reportedly regulated by tyrosine phosphorylation [16, 34], under our conditions pervanadate exerted no significant effects.

Our results thus indicate that both increased phosphorylation levels and prevention of Ca^{2+} inactivation were necessary to couple L-type VDCC to neurotransmitter release, as there was no increment in either QC or MEPP frequency when OA was applied in the EGTA-AM-incubated MNT. However, we cannot exclude the possibility that the fast chelator BAPTA regulates phosphorylation levels of MNT downstream from the L-type VDCC [23].

The relationship between $[Ca^{2+}]_o$ and the amount of neurotransmitter released at vertebrate MNT is linear (in a double-logarithm plot) and slopes of nearly 4 in frogs [14] and 2.7–3.4 in mammals [9, 10] have been reported. Here, we compared the relationship between QC and $[Ca^{2+}]_o$ under conditions in which only P/Q- or L-type VDCC were available to participate in neurotransmitter release and found slope values of 0.7 and 1.4 for neurotransmitter release mediated by P/Q- and L-type VDCC,

respectively in BAPTA-incubated MNT in the presence of OA. Moreover, recent experiments from our group have shown a slope of 2.8 for P/Q-type VDCC-mediated neurotransmitter release in BAPTA-AM-incubated MNT without OA (data not shown). Moreover, there were differences in the EPP latency and rise time between P/Q- and L-type VDCC-mediated EPP. These differences were not due to a decrease in conduction velocity since the delay of the perineural Na^+ current (I_{Na}) is unchanged after the application of high concentration of ω-Aga [48].

Interestingly, Betz and Henkel have described an OA-induced spread of FM1-43 stained synaptic vesicles throughout the MNT of the frog [6]. Likewise, we found OA-induced coupling of L-type VDCC to neurotransmitter release, probably in agreement with a more distant position from active zones distribution of these channels at MNT combined with a higher concentration of distant "OA-disrupted vesicles" available to be liberated. Also, a reduction of the neurotransmitter release after preincubation with OA was observed, agreeing with our low Ca^{2+} co-operativity of P/Q-type VDCC-mediated neurotransmitter release from BAPTA-AM-incubated MNT in the presence of OA. More specific experiments combining optical and ultrastructural techniques may clarify this point. Other cases of multiple VDCC coupling to neurotransmitter release at same synapse have been reported previously [17, 49].

Moreover, the relationship between QC and $[Ca^{2+}]_o$, corresponding to neurotransmitter release mediated by L-type VDCC, showed a drastic and significantly reduction when $[Ca^{2+}]_o$ was increased from 1 to 2 mM, suggesting a typical Ca^{2+}-inactivation feature of L-type VDCC reported in other systems [32, 41]. Calmodulin has been recently described as the Ca^{2+} sensor for Ca^{2+}-dependent inactivation of the L-type VDCC [35, 37, 50]. In fact, calmodulin has a constitutive association with an IQ-like motif on the carboxyl tail of the α_1-subunit of the L-, but also of the N-, P/Q- and R-type VDCC. Interestingly, we found no direct effect of OA on presynaptic Ca^{2+} currents (data not shown), precluding participation of such a mechanism in the process described here. In addition, H-7 attenuated the effect of OA, suggesting the participation of at least PKA and/or PKC. However, more experiments will be necessary to clarify whether other PKs are involved also. This is consistent with a bulk of growing evidence which indicates that Ca^{2+}-calmodulin and PKC obstruct each other in competing for the same domains in many cellular targets [7]. However, more direct measurement of Ca^{2+} influx in these MNT will be necessary clarify this point.

It is also important to note that, in this preparation, calciseptine blocks L-type VDCC in BAPTA-AM-incubated MNT without affecting nerve conduction (data not shown). This peptide has been shown previously to block L-type VDCC in muscle [13] and nerve preparations [40]. It is also well known that the sensitivity of L-type VDCC to calciseptine is tissue dependent [13], being higher in cells of the cardiovascular system than in neural cells.

Many physiological functions have been attributed to the activity of L-type VDCC. In neonatal rats, messenger ribonucleic acid (mRNA) for the α-subunits of L-type VDCC is present in the somata of the rat facial nucleus (i.e. the brainstem nucleus innervating the levator auris longus muscle) motoneurons [36]. In addition, L-type VDCC have been implicated in the process of neurotransmitter release in immature mammalian MNT during development [38], during re-innervation [27] and during functional recovery from intoxication with *botulinum* toxin type-A [39]. These reports might indicate the presence of "silent" but available L-type VDCC during "plastic stages" of MNT. Also, L-type VDCC have been reported to be involved in the release of peptides or neuromodulators [17].

On the other hand, involvement of L-type VDCC in other functions, not associated with neurotransmitter release, have been described. For instance, in hippocampal neurons the Ca^{2+} influx through L-type VDCC causes translocation of calmodulin from the cytoplasm to the nucleus [11]. In cerebellar granule cells L-type VDCC are involved in neuronal development [16].

In conclusion, our results indicate that L-type VDCC can mediate neurotransmitter release under experimental conditions in which intracellular Ca^{2+} is buffered by the fast Ca^{2+} chelator BAPTA and in the presence of OA. This could be a useful model for the understanding of the process of neurotransmitter release by multiple types of VDCC during development.

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References

1. Abdul-Ghani M, Kravitz EA, Meiri H, Rahamimoff R (1991) Protein phosphatase inhibitor okadaic acid enhances transmitter release at neuromuscular junctions. Proc Natl Acad Sci USA 88:1803–1807
2. Adler EM, Augustine GF, Duffy SN, Charlton MP (1991) Alien intracellular calcium chelators attenuate neurotransmitter release at the squid synapse. J Neurosci 11:1496–1507
3. Arenson MS, Gill DS (1996) Differential effects of an L-type Ca^{2+} channels antagonist on activity- and phosphorylation-enhanced release of acetylcholine at the neuromuscular junction of the frog in vitro. Eur J Neurosci 8:437–445
4. Artalejo CR, Rossie S, Perlman RL, Fox AP (1992) Voltage-dependent phosphorylation may recruit Ca^{2+} current facilitation in chromaffin cells. Nature 358:63–66
5. Artalejo CR, Adams ME, Fox AP (1994) Three types of Ca^{2+} channel trigger secretion with different efficacies in chromaffin cells. Nature 367:72–76
6. Betz WJ, Henkel AW (1994) Okadaic acid disrupts clusters of synaptic vesicles in frog motor nerve terminals. J Cell Biol 124:843–854
7. Chakravarthy B, Morley P, Whitfield J (1999) Ca^{2+}-calmodulin and protein kinase Cs: an hypothetical synthesis of their conflicting convergence on shared substrate domains. Trends Neurosci 22:12–16

8. Chik CL, Li B, Karpinski E, Ho AK (1999) Regulation of the L-type Ca^{2+} channel current in rat pinealocytes: role of basal phosphorylation. J Neurochem 72:73–80
9. Cull-Candy SG, Lundh H, Thesleff S (1976) Effects of botulinum toxin on the neuromuscular transmission in the rat. J Physiol (Lond) 260:177–203
10. Cull-Candy SG, Miledi R, Trautmann A, Uchitel OD (1980) On the release of transmitter at normal, myasthenia gravis and myasthenic syndrome affected human end-plates. J Physiol (Lond) 299:621–638
11. Deisseroth K, Heist EK, Tsien RW (1998) Translocation of calmodulin to the nucleus supports CREB phosphorylation in hippocampal neurons. Nature 392:198–202
12. Depetris RS, Urbano FJ, Uchitel OD (1999) Effect of okadaic acid on BAPTA-AM incubated mouse neuromuscular junction (abstract). Proc Physiol Soc:PC113
13. De Weille AR, Schweitz H, Maes P, Tartar A, Lazdunski M (1991) Calciseptine, a peptide isolated from black mamba venom, is a specific blocker of the L-type calcium channel. Proc Natl Acad Sci USA 88:2437–2440
14. Dodge FA, Rahamimoff R (1967) Co-operative action of calcium ions in transmitter release at the neuromuscular junction. J Physiol (Lond) 193:419–432
15. Elhamdani A, Zhou Z, Artalejo CR (1998) Timing of dense-core vesicle exocytosis depends on the facilitation L-type Ca^{2+} channel in adrenal chromaffin cells. J Neurosci 18:6230–6240
16. Evans GJ, Pocock JM (1999) Modulation of neurotransmitter release by dihydropyridine-sensitive calcium channels involves tyrosine phosphorylation. Eur J Neurosci 11:279–292
17. Fossier P, Tauc L, Baux G (1999) Calcium transients, and neurotransmitter release at an identified synapse. Trends Neurosci 22:161–166
18. Frace AM, Hartzell HC (1993) Opposite effects of phosphatase inhibitors on L-type calcium and delayed rectifier currents in frog cardiac myocytes. J Physiol (Lond) 472:305–326
19. Fu WM, Huang FL (1994) L-type Ca^{2+} channel is involved in the regulation of spontaneous transmitter release at developing neuromuscular synapses. Neuroscience 58:131–140
20. Gray DB, Brusés JL, Pilar GR (1992) Developmental switch in the pharmacology of Ca^{2+} channels coupled to acetylcholine release. Neuron 8:1–20
21. Haby C, Larsson O, Islam MS, Aunis D, Berggren PO, Zwiller J (1994) Inhibition of serine/threonine protein phosphatases promotes opening of voltage-activated L-type Ca^{2+} channels in insulin-secreting cells. Biochem J 298:341–346
22. Hidaka H, Inagaki M, Kawamoto S, Sasaki Y (1984) Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry 23:5036–5041
23. Hille B, Billiard J, Babcock DF, Nguyen T, Koh D-S (1999) Stimulation of exocytosis without a calcium signal. J Physiol (Lond) 520:23–31
24. Hubbard JI, Llinás R, Quastel DML (1969) Electrophysiological analysis of synaptic transmission. Edward Arnold, London
25. Hunter T (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signalling. Cell 80:225–236
26. Katz B (1969) The release of neural transmitter substances. (Sherrington lecture series No. 10). Liverpool University Press, Liverpool
27. Katz E, Ferro PA, Weisz G, Uchitel OD (1996) Calcium channels involved in synaptic transmission at the mature and regenerating mouse neuromuscular junction. J Physiol (Lond) 497:687–697
28. Katz E, Protti DA, Ferro PA, Rosato-Siri MD, Uchitel OD (1997) Effects of Ca^{2+} channel blocker neurotoxins on transmitter release and presynaptic currents at the mouse neuromuscular junction. Br J Pharmacol 121:1531–1540
29. Llinás R, Moreno H (1998) Local Ca^{2+} signaling in neurons. Cell Calcium 24:359–366
30. Llinás R, Steinberg IZ, Walton K (1976) Presynaptic calcium currents and their relation to synaptic transmission: voltage clamp study in squid giant synapse and theoretical model for the calcium gate. Proc Natl Acad Sci USA 73:2918–2922
31. Llinás R, Moreno H, Sugimori M, Mohammadi M, Schlessinger J (1997) Differential pre- and postsynaptic modulation of chemical transmission in the squid giant synapse by tyrosine phosphorylation. Proc Natl Acad Sci USA 94:1990–1994
32. McDonald TF, Pelzer S, Trautwein W, Pelzer DJ (1994) Regulation and modulation of calcium channels in cardiac, skeletal, and smooth muscle cells. Physiol Rev 74:365–507
33. Neher E (1998) Vesicle pools and Ca^{2+} microdomains: new tools for understanding their roles in neurotransmitter release. Neuron 20:389–399
34. Ogura T, Shuba LM, McDonald TF (1999) L-type Ca^{2+} current in guinea pig ventricular myocytes treated with modulators of tyrosine phosphorylation. Am J Physiol 276:H1724–H1733
35. Peterson BZ, DeMaria CD, Yue DT (1999) Calmodulin is the Ca^{2+} sensor for Ca^{2+}-dependent inactivation of L-type calcium channels. Neuron 22:549–558
36. Plant TD, Schirra C, Katz E, Uchitel OD, Konnerth A (1998) Single-cell RT-PCR and functional characterization of Ca^{2+} channels in motoneurons of the rat facial nucleus. J Neurosci 18:9573–9584
37. Qin NQ, Olcese R, Bransby M, Lin T, Birnbaumer L (1999) Ca^{2+}-induced inhibition of the cardiac Ca^{2+} channel depends on calmodulin. Proc Natl Acad Sci USA 96:2435–2438
38. Rosato-Siri MD, Uchitel OD (1999) Calcium channels coupled to neurotransmitter release at neonatal rat neuromuscular junctions. J Physiol (Lond) 514:533–540
39. Santafé MM, Urbano FJ, Lanuza MA, Uchitel OD (2000) Multiple types of calcium channels mediate transmitter release during functional recovery of *botulinum* toxin type-A-poisoned mouse motor nerve terminals. Neuroscience 95:227–234
40. Satoh Y, Hirashima N, Tokumaru H, Takahashi MP, Kang J, Viglione MP, Kim YI, Kirino Y (1998) Lambert-Eaton syndrome antibodies inhibit acetylcholine release and P/Q-type Ca^{2+} channels in electric ray nerve endings. J Physiol (Lond) 508:427–438
41. Schuhmann K, Romanin C, Baumgartner W, Groschner K (1997) Intracellular Ca^{2+} inhibits smooth muscle L-type Ca^{2+} channels by activation of protein phosphatase type 2B and direct interaction with the channel. J Gen Physiol 110:503–513
42. Sena CM, Santos RM, Boarder MR, Rosário LM (1999) Regulation of Ca^{2+} influx by a protein kinase C activator in chromaffin cells: differential role of P/Q- and L-type Ca^{2+} channels. Eur J Pharmacol 366:281–292
43. Tokuda M, Hatase O (1998) Regulation of neuronal plasticity in the central nervous system by phosphorylation and dephosphorylation. Mol Neurobiol 17:137–156
44. Tsien RY (1981) A non-disruptive technique for loading calcium buffers and indicators into cells. Nature 290:527–528
45. Turner KM, Burgoyne RD, Morgan A (1999) Protein phosphorylation and the regulation of synaptic membrane traffic. Trends Neurosci 22:459–464
46. Tymianski M, Charlton MP, Carlen PL, Tator CH (1994) Properties of neuroprotective cell-permeant Ca^{2+} chelators: Effects on $[Ca^{2+}]_i$ and glutamate neurotoxicity in vitro. J Neurophysiol 72:1973–1992
47. Uchitel OD (1997) Toxins affecting calcium channels in neurons. Toxicon 35:1161–1191
48. Urbano FJ, Uchitel OD (1999) L-type calcium channels unmasked by cell-permeant Ca^{2+}-buffer at mouse motor nerve terminals. Pflügers Arch 437:523–528
49. Wu L-G, Westenbroek RE, Borst GG, Catterall WA, Sakmann B (1999) Calcium channel types with distinct presynaptic localization couple differentially to transmitter release in single calyx-type synapses. J Neurosci 19:726–736
50. Zühlke RD, Pitt GS, Deisseroth K, Tsien RW, Reuter H (1999) Calmodulin supports both inactivation and facilitation of L-type calcium channels. Nature 399:159–162