Cyclosporin and Timothy syndrome increase mode 2 gating of CaV1.2 calcium channels through aberrant phosphorylation of S6 helices

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Calcium channels in the plasma membrane rarely remain open for much more than a millisecond at any one time, which avoids raising intracellular calcium to toxic levels. However, the dihydropyridine-sensitive calcium channels of the CaV1 family, which selectively couple electrical excitation to endocrine secretion, cardiovascular contractility, and neuronal transcription, have a unique second mode of gating, “mode 2,” that involves frequent openings of much longer duration. Here we report that two human conditions, cyclosporin neurotoxicity and Timothy syndrome, increase mode 2 gating of the recombinant rabbit CaV1.2 channel. In each case, mode 2 gating depends on a Ser residue at the cytoplasmic end of the S6 helix in domain I (Ser-439, Timothy syndrome) or domain IV (Ser-1517, cyclosporin). Both Ser reside in consensus sequences for type II calmodulin-dependent protein kinase. Pharmacologically inhibiting type II calmodulin-dependent protein kinase or mutating the Ser residues to Ala prevents the increase in mode 2 gating. We propose that aberrant phosphorylation, or “phosphorylopathy,” of the CaV1.2 channel protein contributes to the excitotoxicity associated with Timothy syndrome and with chronic cyclosporin treatment of transplant patients.

calcium/calmodulin-dependent protein kinase type II | calcineurin | dihydropyridine | excitotoxicity

Calcium regulates many fundamental cellular processes through calcium-binding proteins, which have affinities for calcium in the micromolar range (1). However, sustained increases in cytoplasmic calcium are cytotoxic. To avoid cytotoxicity, voltage-activated calcium channels rarely remain open for more than a millisecond or two at any one time. Nevertheless, one class of calcium channels, the dihydropyridine-sensitive CaV1 family of channels, which are uniquely responsible for coupling electrical excitation to endocrine secretion, cardiac and vascular muscle contractility, and neuronal transcription (2), have a second mode of gating that involves frequent openings of much longer duration (3). “Mode 2” gating was discovered as a pharmacological effect of the dihydropyridine Bay K8644 (4), but subsequent work has implicated reversible protein phosphorylation of the channel by calcium/calmodulin-dependent protein kinase type II (CaMKII) in the regulation of mode 2 gating in cells that were not treated with dihyropyridines (5). Although the molecular mechanism of this effect and its physiological consequences are only beginning to be investigated (6–9), it is likely that unregulated mode 2 gating would be cytotoxic because the individual channel openings in mode 2 are >10 times longer on average than the openings in mode 1.

Many human diseases are produced by inherited mutations in ion channel proteins (10), but most “channelopathies” result from mutations that produce loss of channel function. Two human neurological disorders are associated with calcium cytotoxicity, which could result from a gain of function by calcium-selective channels, but their relationship to mode 2 gating has not been investigated. One disorder is the neurotoxic effects of cyclosporin, an inhibitor of the calcium/calmodulin-dependent protein phosphatase IIB calcineurin (11), which regulates transcription in lymphocytes (12) but has also been reported to inhibit dihydropyridine-sensitive calcium channels (13–15). Cyclosporin is given to transplant patients to inhibit the immune response and prevent rejection of the transplanted tissue; however, chronic cyclosporin use has unexplained neurotoxic side effects that could result in part from disinhibition of calcium channels (16). Another human disorder with more devastating neurological and cardiological consequences is Timothy syndrome, which was recently shown to result from the mutation G436R at the cytoplasmic end of the IS6 transmembrane helix encoded by the human CaV1.2 gene (17). Functionally this mutation also increases macroscopic calcium currents by slowing inactivation.

We recently reported that inhibition of phosphoprotein Ser/Thr phosphatases with the microbial toxin okadaic acid stimulates mode 2 activity of recombinant rabbit cardiac CaV1.2 channels as effectively as Bay K8644 (18). We postulated a physical link between the molecular mechanisms responsible for the two effects. Specifically, we noted that the IS6 transmembrane helix encoded by CaV1 genes might potentially couple dihydropyridine binding in the bilayer and protein phosphorylation in the cytoplasm to the same gating mechanism through Ser-1142 in the pore loop helix of domain III, which itself is unlikely to be phosphorylated. However, we noted that only concentrations of okadaic acid >500 nM stimulated mode 2 activity. This observation implicates calcineurin in regulating mode 2 activity because all of the other okadaic acid-sensitive Ser/Thr phosphatases are blocked by lower concentrations of okadaic acid (19).

Here we show that cyclosporin, a more selective inhibitor of calcineurin (11), increases mode 2 activity of CaV1.2 channels through a CaMKII-dependent mechanism involving Ser-1517 at the cytoplasmic end of IS6. We also show that the Timothy syndrome mutation, which is homologous to G436R in the rabbit CaV1.2 gene, produces spontaneous mode 2 gating that appears to depend on CaMKII-dependent protein phosphorylation of Ser-439 at the end of IS6. Thus, the neurotoxicity associated with Timothy syndrome and the neurotoxicity of chronic cyclosporin treatment in transplant patients might result from unregulated mode 2 gating produced by aberrant hyperphosphorylation of the channel protein.

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Abbreviation: CaMKII, calcium/calmodulin-dependent protein kinase type II.
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Results
In electrophysiological studies conducted with exogenous calcium buffers, spontaneous mode 2 gating in the absence of dihydropyridines is rarely observed (20, 21). To avoid depleting intracellular calcium, we recorded calcium channel activity in cell-attached patches on cells bathed in a high-potassium salt solution (see Materials and Methods) that contained 0.1 mM calcium but no exogenous calcium buffers. Under these conditions, cyclosporin promotes mode 2 gating of native dihydropyridine-sensitive calcium channels in primary embryonic rat cortical neurons and clonal rat pituitary cells (Fig. 1). To quantify the differences between mode 1 gating, which consists of short bursts of very brief openings, and mode 2 gating, which consists of much longer openings, the duration of every recorded opening event from each patch was collected in a histogram, which was fit with two exponentials (demarcated by dashed lines in the figures). In every case the best fits were obtained with two time constants representing the average open duration of mode 1 ($\tau_1 < 0.5$ ms) and mode 2 ($\tau_2 > 3.0$ ms). The percentage of the events that fit into each distribution provides a measure of the time spent in mode 1 or mode 2 gating. Although the spontaneous rate of mode 2 gating under control conditions was significantly higher in the neurons ($17 \pm 3\%$, $n = 3$) than in GH4C1 cells ($7 \pm 2.5\%$, $n = 5$), cyclosporin ($20 \mu M$) produced dramatic increases in mode 2 gating in every patch (Fig. 1E and F), increasing mode 2 frequency to $31 \pm 2.4\%$ in neurons ($n = 3$) and $23 \pm 4\%$ in GH4C1 cells ($n = 5$).

We hypothesize that the effect of inhibiting calcineurin with cyclosporin is mediated by abnormal hyperphosphorylation of the channel complex because it was prevented with $10 \mu M$ 1-[N,O-bis-(5-isouquinolinesulfonyl)]-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), a selective inhibitor of CaMKII (22), which is the Ser/Thr kinase believed to be responsible for mode 2 gating (5). In GH4C1 cells, $10 \mu M$ KN-62 reduced the proportion of mode 2 openings in cyclosporin from $23 \pm 4\%$ to $4 \pm 2.1\%$ ($n = 5$) (Fig. 1D and F) without altering the mean value of either $\tau_1$ or $\tau_2$ or the overall level of activity (Fig. 1C and D). Thus, KN-62 is not inhibiting mode 2 activity by acting as a pore blocker. Although mode 2 activity never became the predominant mode of gating, mode 2 openings last $>10$ times longer on average than mode 1 openings. Consequently, a 10% increase in the proportion of mode 2 openings would produce a $>100%$ increase in calcium entry, which might contribute to the observed neurotoxicity of chronic cyclosporin use in transplant patients.

Timothy syndrome is a multiorgan disorder associated with calcium toxicity in humans that was recently shown to result from a specific mutation, G406R, at the cytoplasmic end of IS6 in the $\alpha_1C$ subunit of human CaV1.2 (17). Macroscopic calcium currents through mutant channels expressed in mammalian fibroblasts decayed more slowly during sustained depolarization, which was interpreted as a decrease in inactivation (17). However, when we introduced the corresponding mutation, G436R, in rabbit CaV1.2 and expressed it in baby hamster kidney 6 (BHK6) cells, we observed a decrease in unitary channel conductance from 22 to 14 pS (data not shown) and an increase in spontaneous mode 2 gating (Fig. 2B and E). In patches expressing the G436R mutation, $12.4 \pm 3\%$ of the opening events were mode 2 with an average duration of $8.5 \pm 1.1$ ms ($n = 27$). Such mode 2 gating could produce the apparent decrease in macroscopic current inactivation and consequent excitotoxicity reported for Timothy’s syndrome. In contrast, mode 2 openings of recombinant wild-type rabbit CaV1.2 channels (Fig. 2A and E) were significantly shorter ($3.8 \pm 1.3$ ms) and less frequent ($3.9 \pm 1.9\%$) ($n = 8$). The increase in mode 2 activity of the G436R mutant was observed without adding any dihydropyridines or phosphatase inhibitors. Nevertheless, we noted that the G436R mutation created a CaMKII consensus site surrounding Ser-439, so we hypothesized that phosphorylation of Ser-439 might be responsible for the unregulated mode 2 activity.

In support of this hypothesis, we observed that either inhibition of CaMKII with KN-62 (Fig. 2C and E) or the mutation

![Fig. 1. Inhibition by cyclosporine (CsA) of the calcium/calmodulin-dependent protein phosphatase 1B calcineurin results in an increase of long, mode 2 openings of native $\alpha_1$-type channels in neurons and pituitary cells. Shown are representative records from cell-attached patches at 0 mV and open time histograms for all open events from one patch for dissociated primary embryonic rat cortical neurons (A), neurons treated with $20 \mu M$ cyclosporin (B), GH4C1 cells treated with $20 \mu M$ cyclosporin (C), and GH4C1 cells treated with $20 \mu M$ cyclosporin and $10 \mu M$ KN-62 (D). The dashed lines in the channel-open time histograms are maximum-likelihood fits by the sum of two exponentials, which represent short ($\tau_1$) and long ($\tau_2$) open times. (E and F) Summaries of the values for the frequency of mode 2 openings from the indicated cell type and treatment. Con, control.](image-url)
S439A (Fig. 2D and E) completely eliminated the increase in spontaneous mode 2 activity produced by G436R. Of course, KN-62 also suppresses spontaneous long openings of the native channels (Fig. 1). However, the S439A mutation on the G436R background reduced spontaneous mode 2 open duration (3.7 ± 0.7 ms) and frequency (2.5 ± 0.7%) to the same levels as wild type (n = 20) (Fig. 2C). Thus, we propose that Timothy syndrome, like the neurotoxic effects of cyclosporin, might represent a “phosphorylopathy” of the CaV1.2 calcium channel.

Biochemical analysis confirmed that a peptide based on the CaV1.2 sequence surrounding G346R is an excellent substrate for CaMKII (Fig. 3). Radiolabeled phosphate was incorporated into the peptide in stoichiometric amounts by purified CaMKII (Fig. 3A), and mass spectrometry (MS) confirmed that the phosphate was incorporated only at Ser-439 (Fig. 3C). In contrast, purified CaMKII was unable to incorporate any phosphate into the wild-type sequence with Gly-436, although Ser-439 was present (Fig. 3A). By measuring the dependence of the initial rate of phosphorylation on ATP and peptide concentration (Fig. 3B), we estimated the $K_m$ and $V_{max}$ for CaMKII phosphorylation of the peptide. To calibrate our experimental system, we also studied CaMKII phosphorylation of autocamtide, a peptide from CaMKII that is the best characterized peptide substrate for purified CaMKII described to date (23). Although the $K_m$ of purified CaMKII for the G436R peptide (152 ± 92 M) was signifi-
Peptide has a higher affinity for CaMKII (the G436R peptide and a comparable V each condition. CsA, cyclosporin; Bay K, Bay K8644.

Frequency of mode 2 openings from the indicated number of patches under each condition. CsA, cyclosporin; Bay K, Bay K8644.

Fig. 4. Mutation of Ser-1517 at the cytoplasmic end of IVS6 decreases the frequency of mode 2 openings. Shown are representative records and open time histograms for all events from one patch for S1517A (A), S1517A treated with 20 μM cyclosporin (B), and S1517A treated with 100 nM (−)Bay K8644 (C). (D) Summary of the values for the long open times, τo2, as well as the frequency of mode 2 openings from the indicated number of patches under each condition. CsA, cyclosporin; Bay K, Bay K8644.

Significantly lower than the K_m for autocamtide (8.1 μM), the V_max was almost identical: 8.9 μmol·min^{−1}·mg^{−1} for the G436R peptide versus 9.8 μmol·min^{−1}·mg^{−1} for autocamtide.

The association of spontaneous mode 2 gating with the introduction of a CaMKII consensus site at the cytoplasmic end of IS6 prompted us to look for a comparable sequence that could explain mode 2 opening in native channels when calcineurin is blocked. Ser-1517 at the cytoplasmic end of IVS6 occupies the homologous spatial position as Ser-439 at the cytoplasmic end of IS6. Ser-1517 is also located in a highly conserved CaMKII homologous spatial position as Ser-439 at the cytoplasmic end of IVS6 prompted us to look for a comparable sequence that could explain mode 2 opening in native channels when calcineurin is blocked. Ser-1517 at the cytoplasmic end of IVS6 occupies the homologous spatial position as Ser-439 at the cytoplasmic end of IS6. Ser-1517 is also located in a highly conserved CaMKII consensus sequence (Fig. 3D). Although the corresponding Ser-1517 peptide is not phosphorylated stoichiometrically by CaMKII in vitro (Fig. 3A), MS revealed that the peptide is phosphorylated only on Ser-1517 (Fig. 3D). Furthermore, the peptide has a higher affinity for CaMKII (K_m = 26.5 μM) than the G436R peptide and a comparable V_max (8.1 μmol·min^{−1}·mg^{−1}). Mutation of Ser-1517 to Ala had no significant effect on the open durations or frequencies of either mode 1 or mode 2 under control conditions (Fig. 4A and D, compare to Fig. 2A and E, respectively), but S1517A completely prevented any increase in mode 2 gating in the presence of cyclosporin (n = 5) (Fig. 4B and D). Nevertheless, S1517A does not prevent the (−) enantiomer of Bay K8644 from dramatically increasing the duration (7.0 ± 1.3 ms) and frequency (23 ± 2.6%) of mode 2 activity (n = 6) (Fig. 4C and D). Thus, the S1517A mutation prevents only cyclosporin and not dihydropyridines from increasing mode 2 activity, which is consistent with the hypothesis that phosphorylation of Ser-1517 is a critical event for the mode 2 activity of native channels.

Discussion

We have shown that the calcineurin inhibitor cyclosporin and the mutation associated with Timothy syndrome, G436R, both stimulate mode 2 gating in recombinant rabbit cardiac CaV1.2 channels, which were expressed heterologously in embryonic hamster kidney cells and recorded in the absence of any exogenous calcium buffers. Cyclosporin also increases mode 2 gating of native dihydropyridine-sensitive calcium channels in primary dissociated cortical neurons and clonal rat pituitary cells. The stimulation of mode 2 activity is blocked by the selective CaMKII inhibitor KN-62 and by mutating the putative phosphoacceptors Ser-439 and Ser-1517 in the CaMKII consensus sequences where the IS6 and IVS6 transmembrane helices are predicted to enter the cytoplasm (18). Furthermore, purified CaMKII phosphorylates these peptides in vitro almost as effectively as the model substrate, autocamtide. The simplest hypothesis consistent with all these results posits that phosphorylation of Ser-1517 at the cytoplasmic end of the IVS6 helix by the calcium/calmodulin-dependent protein kinase produces a force that opposes closing of native channels (Fig. 5). This effect is reversed by dephosphorylation with the calcium/calmodulin-dependent phosphatase, calcineurin. When calcineurin is inhibited with cyclosporin, increased mode 2 gating contributes to neurotoxicity through elevated cytoplasmic calcium levels. This neurotoxicity is exacerbated in Timothy’s syndrome by the introduction of a second homologous CaMKII phosphorylation site on Ser-439 at the end of the IS6 helix, which appears to be less susceptible to dephosphorylation because it produces spontaneous mode 2 activity without cyclosporin.

Regulation of native mode 2 gating at the single-channel level has been reported with purified CaMKII on cardiac myocytes (5) and with calcineurin on vascular smooth muscle (14), but the sites of action were not identified. Here we demonstrate similar effects on neurons and electrically excitable endocrine cells and on recombinant CaV1.2 channels. We also identify a putative phosphorylation site at Ser-1517 for the effects of these enzymes, although it remains to be shown whether this site is reversibly phosphorylated on intact channels in vivo. Nevertheless, in what is otherwise a remarkable coincidence, the homologous site in CaV1.2 at Ser-439 in IS6 is required for the spontaneous, CaMKII-dependent mode 2 activity that results from introducing the Timothy syndrome mutation at G436 in rabbit CaV1.2. Introduction of the basic Arg residue at Gly-436 not only increases mode 2 gating, it converts the peptide surrounding Ser-439 into an excellent substrate for CaMKII in vitro (Fig. 3).

Of course, there are many more elaborate explanations for this data that we have not tested involving other proteins that have been reported to modulate CaV1 activity (2), including the β subunit, calmodulin, the cytoskeleton, and other protein kinases and phosphatases. However, much of the previous work on CaV1 regulation was not conducted at the single-channel level and often used exogenous calcium buffers, making it difficult to infer changes in gating mode or enzyme availability or to rule out changes produced by channel trafficking. Furthermore, it remains to be explained why a homologous sequence in IVS6 of CaV2 family channels is not sufficient to stimulate mode 2 openings. CaMKII has been reported to stimulate the activity of recombinant CaV3.2 channels by phosphorylating the cytoplasmic linker connecting IS6 to IIIS1 (24), but it had no effect on channel open time. Therefore, given the increasing importance of CaMKII, calcineurin, and dihydropyridine-sensitive calcium
Neurons were isolated from cortex hemispheres, which were dissected from 19-day-old rat embryos and collected in a conical tube with 1 ml of Hanks’ balanced salt solution. Neurons were suspended by gentle trituration (20 times) using a Pasteur pipette. The cells were allowed to settle for 3 min then spun at 200 × g for 1 min. Neurons were plated at the desired concentration in neurobasal medium (GIBCO) on 12-mm polylysine-coated coverslips and kept in 5% CO2 at 34°C for 7–10 days before the patch-clamp measurements.

Site-Directed Mutations in α1C Subunit. Mutations in rabbit α1C (GenBank accession no. X15539) were introduced into short cassettes by PCR using a QuikChange XL site-directed mutagenesis kit (Stratagene) and respecified into the full-length α1C cDNA in the expression vector pKNH. Each clone was sequenced in both directions to confirm mutation and integrity. Baby hamster kidney cells were transfected with the indicated α1C construct and GFP using Lipofectamine according to the manufacturer’s instructions.

Electrophysiology. Unitary barium currents were recorded under voltage-clamp in the cell-attached configuration as described in ref 18. The patch pipettes were filled with 90 mM BaCl2/10 mM Hepes, with the pH adjusted to 7.4 with tetrathylammonium hydroxide. All experiments were performed at room temperature (20–24°C) on cells bathed in a high concentration of potassium to zero the membrane potential. The extracellular bath solution consisted of 145 mM KCl, 2 mM MgCl2, 0.1 mM CaCl2, and 10 mM Hepes, with the pH adjusted to 7.4 with KOH, which contained 0.1 mM calcium to avoid depleting the cells’ calcium stores. Patches on neurons and GH3 cells were held at −50 mV and stepped to 0 mV for 0.1–1.0 sec at 5-s intervals. Patches on baby hamster kidney cells expressing recombinant channels were held at 0 mV, and activity was recorded continuously or in 10-s blocks separated by 5-s intervals. All of the records were acquired at 10 kHz and filtered at 1–2 kHz. All single-channel records from each patch were analyzed, collected into histograms of open duration, and fit with two exponentials with TACFIT software (Bruxton, Seattle). Averaged data are presented as mean ± SE. In Figs. 1, 2, and 4, differences were evaluated with the Student t test (single asterisk, P = 0.05; double asterisk, P = 0.01).

Peptide Phosphorylation and Analysis. Rat CaMKII was expressed in human embryonic kidney 293 (HEK293) cells and purified as described in ref. 23. CaV1.2 peptides were made by the Peptide Synthesis Facility in the Department of Microbiology and Immunology at the University of North Carolina and used at concentrations between 25 and 250 μM. Peptide phosphorylations were performed at 30°C in 50 mM Hepes, pH 7.5/1 mM DTT/5 mM MgCl2/0.1% Tween 80/0.2 mg/ml BSA. ATP (Boehringer Mannheim) was varied between 0.1 and 1 mM. Reactions were initiated by adding 10 nM CaMKII, allowed to proceed for 4 min, and terminated by spotting on P-81 filters. The filters were washed in 3 mM phosphoric acid, and scintillation was counted. Under these conditions, <5% of substrate was consumed in each reaction. Kinetic constants were derived from linear regression analysis of double reciprocal primary and secondary plots for a general two-substrate reaction. To phosphorylate the peptides to high stoichiometry, 0.2 mM peptide was combined with 0.6 mM ATP and 20–40 μCi (1 Ci = 37 GBq) [γ32-P]ATP (Amersham Pharmacia). Four additions of 5 nM CaMKII were made to the reaction over the course of 1 h. Mol of phosphate incorporated per mol of peptide was determined by spotting 5 μl of the reaction mix at various time points on P-81 filters followed by washing and counting as above. Phosphorylated CaV1.2 peptides were isolated from phosphorylation re- actions and prepared for MS by passing the reaction mixture over Bio-Rad AG1-X ion-exchange resin equilibrated and eluted with channels in neuronal plasticity and gene regulation (25–29), our hypothesis, which is consistent with newer structural data emphasizing the importance of the S6 helices in gating (30), provides a simple, testable mechanism for integrating many old and new observations on calcium channel regulation by calcium and reversible protein phosphorylation.

Materials and Methods

Cells. Embryonic hamster kidney 6 cells, which have been engineered to stably express rabbit CaV1 β1a and α1S/8 subunits, (31) were maintained in gentamycin and DMEM with 10% FBS. Rat pituitary GH3 cells were maintained in Ham’s F-12/DMEM with 10% calf serum and penicillin–streptomycin at 37°C in 5% CO2. Neurons were isolated from cortex hemispheres, which
30% acetic acid. Peptides were then desalted by passage over Sephadex G-25 in 10 mM Hepes, pH 7.5, and purified by RP-HPLC using a C-18 column eluted with a 0–30% gradient of acetonitrile in water. Phosphorylated residues were identified by MS.

**MS and Tandem MS (MS/MS) of Phosphopeptides.** Peptide samples were desalted with C₁₈ ZipTips (Millipore) by following the manufacturer’s recommendations, except the tips were eluted with 5 μl of 30:70 (vol/vol) 0.2% formic acid:acetonitrile. For MALDI-TOF MS and MS/MS analyses, 0.3-μl samples were spotted onto a 192-sample stainless steel MALDI plate and mixed on target with 0.3 μl of 33% saturated a-cyanohydroxycinnamic acid. In mass spectrometric analyses, both MS and MS/MS were then performed on an Applied Biosystems 4700 Proteome Analyzer in the positive ion and reflector modes. The MS was externally calibrated by using a suite of standard peptides (corticotropin clip 1-17, corticotropin clip 18-39, angiotensin I, angiotensin II, bombesin, somatostatin, and substance P) and the MS/MS calibrated externally using the fragment ions of the angiotensin I M+H ion. A focus mass of m/z 2000 is used for the MS acquisition. For the MS/MS, 1,000 V is used for the collision energy and argon is used as the collision gas with a recharge threshold set at 1.0 × 10⁻⁷ torr (1 torr = 133 Pa). Electrospray ionization MS and MS/MS analyses were performed with a Q-Tof Ultima mass spectrometer using flow injection from a pressurized bomb at a flow rate of ~300 nl/min. The instrument was calibrated with the fragment ions of the rennin substrate tetradecapeptide ion m/z 586.8. Mass spectrometer settings for MS analyses included a capillary voltage of 3.8 kV, a cone voltage of 90 V, collision energy of 10 V, a radio frequency lens set at 110 V, and a cone gas flow of 50 liters/h. Similar settings were used for MS/MS analyses, except that the collision energy ranged from 20 to 35 V. The site(s) of phosphorylation was determined from electrospray ionization MS/MS and MALDI-TOF MS/MS by using the resulting b and y series ions with particular focus directed toward the presence and/or absence of phosphorylated b and y ions and/or to b and y ions showing the loss of phosphoric acid (H₃PO₄).

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