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Differential regulation of mammalian brain-specific proline transporter by calcium and calcium-dependent protein kinases

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1 This study examined the role of $[Ca^{2+}]_I$ and Ca^{2+}-dependent kinases in the modulation of high-affinity, mammalian brain-specific L-proline transporter (PROT).

2 β-PMA (phorbol 12-myristate 13-acetate), an activator of protein kinase C (PKC), inhibits PRO uptake, and bisindolylmalemide I (BIM), a potent PKC inhibitor, prevents β-PMA inhibition. Down-regulation of PKC by chronic treatment with β-PMA enhances PROT function indicating PROT regulation by tonic activity of PKC.

3 Thapsigargin, which increases $[Ca^{2+}]_I$ levels by inhibiting Ca^{2+}-ATPase, inhibits PROT and exhibits additive inhibition when co-treated with β-PMA. KN-62, a Ca^{2+}/calmodulin-dependent kinase II (CaMK II) inhibitor, but not BIM (a PKC inhibitor) prevents the inhibition by thapsigargin. These data suggest that PKC and CaMK II modulate PROT and that thapsigargin mediates its effect *via* CaMK II.

4 Thapsigargin raises $[Ca^{2+}]_I$ and increases PRO-induced current on a second time scale, whereas the inhibitory effect of thapsigargin occurs only after 10 min of treatment. These data suggest that Ca^{2+} differentially regulate PROT: Ca^{2+} initially enhances PRO transport but eventually inhibits transport function through CaMK II pathway.

5 Ca^{2+}-induced stimulation exemplifies the acute regulation of a neurotransmitter transporter, which may play a critical role in the profile of neurotransmitters during synaptic transmission.
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Abbreviations: GGFL, gly-gly-phe-leu; HP cells, HEK-cells stably transfected with proline transporter; PRO, L-proline; PROT, brain specific high-affinity proline transporter.

Introduction

The mammalian high affinity L-proline transporter (PROT) cloned from both rat and human brain (Fremeau *et al*., 1992; Shafqat *et al*., 1995) belongs to a high affinity Na^+- and Cl^--dependent plasma membrane transporter gene family. This family includes transporters for neurotransmitters GABA (γ-aminobutyric acid), NE (norepinephrine), 5-HT (serotonin), DA (dopamine), and glycine, and for osmolytes (betaine and taurine) and metabolites (creatine) (Shafqat *et al*., 1993). The substrate specificity, ionic dependence and kinetics of PROT are distinctly different from the other widely expressed mammalian Na^+-dependent plasma membrane transporters that transport L-proline (PRO), including the intestinal brush border 'imino' carrier (Stevens *et al*., 1984) and system 'A' and system 'ASC' neutral amino acid carriers (Christensen, 1990). Recent findings indicate that enkephalins competitively inhibit mammalian PROT through a direct interaction with the transporter protein at or near substrate binding site (Fremeau *et al*., 1996). Intra-cerebral injections of PRO are toxic (Nadler *et al*., 1988) and disrupt memory (Cherkin *et al*., 1976). These excitotoxic properties of PRO may contribute to the pathophysiology of type II hyperprolinemia, a genetic disorder characterized by elevated levels of PRO in plasma and cerebrospinal fluid and a high incidence of childhood seizures (Phang & Scriver, 1989). Furthermore the expression of PROT in sub-populations of putative glutamatergic neurons in rat brain (Fremeau *et al*., 1992; Nadler *et al*., 1992; Velaz-Faircloth *et al*., 1995) suggests a role for this transporter in modulating excitatory neurotransmission at specific glutamatergic synapses. However, very little research has been carried out on the regulation of PROT.

Ca^{2+}-dependent protein kinases are important components of the cellular signal transduction system and are known to regulate several membrane-bound ion channels and carrier proteins. The two main groups of Ca^{2+}-dependent protein kinases are: (1) Ca^{2+} and phospholipid (PL)-dependent protein kinase (PKC) and (2) Ca^{2+}/calmodulin-dependent protein kinase (CaMK). Isoforms of PKCs have been classified into three groups based on their structural and biochemical characteristics: (1) classical or conventional PKCs (cPKCs) activated by Ca^{2+}, PL and diacylglycerol (DG) or phorbol esters, (2) new PKCs (nPKCs) that do not require Ca^{2+} but are activated by PL and DG, or phorbol esters and (3) atypical PKCs (aPKCs) that depend only on PL but are not affected by Ca^{2+}, DG or phorbol ester (Nashizuka, 1992; Casabona, 1997). Both PKC- and CaMK-dependent regulation of plasma-membrane transporters, such as the serotonin transporter (SERT) and the taurine transporter, are well established (Jayanthi *et al*., 1994; Tchoumkeu-Nzouessa & Rebel, 1996; Qian *et al*., 1997; Ramamoorthy *et al*., 1998) Although several studies have attempted to address the physiological role for brain specific PROT through biochemical, immunological and genetic analyses (Nadler *et al*., 1992; Crump *et al*., 1997; Kleven *et al*., 1997), there has been no study of regulation. One study has reported on the regulation of PRO uptake (not carried by brain-specific PROT) in renal brush-border membranes by Ca^{2+}-dependent protein kinases (Zelikovic & Przekwas, 1995).

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We have previously demonstrated that neurotransmitter transporters, such as norepinephrine and serotonin transporters (NET and SERT), exhibit substantial substrate-induced currents (Galli *et al*., 1995; 1996; 1997; 1998; Petersen & DeFelice, 1999). Furthermore, SERT-associated currents in mammalian cells are modulated by PKC in parallel with transport function (Qian *et al*., 1997). Recently we have shown that HEK-293 cells stably transfected with rat brain-specific high affinity PROT cDNA (HP cells) take up PRO; des-tyr-leucine enkephalin (GGFL) blocks this uptake by competitive inhibition (Galli *et al*., 1999). Under whole-cell voltage-clamp, HP cells generate a GGFL-sensitive current in response to PRO; however a biophysical analysis suggested that it behaves as a classical, fixed stoichiometry, coupled transporter (Galli *et al*., 1999). The present study focuses on the differential regulation of PROT (both classical transport function and channel-like properties) by Ca^{2+} and Ca^{2+}-dependent protein kinases. We discuss the possible implications of acute regulation of PROT by Ca^{2+}, and the role of acute regulation in synaptic transmission.

Methods

Chemicals and reagents

α- and β- Phorbol 12-myristate 13-acetate (α-PMA & β-PMA) and 1-[N,O-bis-(5-Isoquinolinesulphonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62) are from Alexis Biochemicals, San Diego, CA, U.S.A. Thapsigargin (sesquiterpene lactone) from Molecular Probes, Netherlands. Bisindolylmalemide I (BIM) from Calbiochem, La Jolla, CA, U.S.A. Des-tyr-leucine enkephalin (GGFL or gly-gly-phe-leu), a tetrapeptide is from Sigma Chemical Co, St. Louis, MO, U.S.A. [^{3}H]-L-proline (specific activity, 80.0 Ci mmol^{-1}) is from NEN, Boston, MA, U.S.A. All other reagents are of analytical grade.

Culture of human embryonic kidney (HEK -293) cells stably transfected with rat PROT cDNA (HP cells)

HP cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% foetal bovine serum, 2 mM L-glutamine and penicillin (100 u ml^{-1}) and streptomycin (100 μg ml^{-1}). Trypsin-released cells were seeded in 24 well cell culture plates (pretreated with poly-D-lysin 0.1 mg ml^{-1}) at 100–150 thousand cells per well and allowed to grow for 48 h in an atmosphere of 95% air/5% CO_2 before the uptake measurements were done.

Treatment of HP cells with thapsigargin and kinase modulators

Treatments were done with (in μM) β-PMA 1, thapsigargin 5, BIM 1 and KN-62 25 in Krebs-Ringer-HEPES (KRH) buffer pH 7.4 ((in mM) NaCl 120, KCl 4.7, $CaCl_2$ 2.2, HEPES 10, $MgSO_4$ 1.2, KH_2PO_4 1.2, Tris 5 and D-glucose 10) for 1 h at 37°C before the uptake is carried out. BIM and KN-62 were added 20 min before the addition of β-PMA or thapsigargin in the cotreatment experiments. In time course studies β-PMA or thapsigargin is added along with labelled PRO and uptake of PRO is measured over a time period of 30 s to 1 h. PKC is down-regulated by treating the HP cells with 1 μM β-PMA for overnight (12–16 h), and the effect of 1 μM β-PMA or 5 μM thapsigargin were tested for their acute effect by incubating the cells (which are treated with or without β-PMA for overnight) with fresh 1 μM β-PMA or 5 μM thapsigargin for 1 h at 37°C before carrying out PRO uptake experiments.

Uptake measurements

Uptake measurements were performed in duplicates by incubating the HP cells for 20 min at 37°C, with [^{3}H]-L-proline in 0.5 ml of KRH buffer. Assays were terminated by removing the radiolabelled PRO and by rapid washings of cells three times with 1 ml ice cold KRH buffer. Cells were solubilized with 1 ml of Optiphase scintillant (Wallac, Gaithersburg, MD, U.S.A.) and accumulated radioactivity quantified by direct scintillation

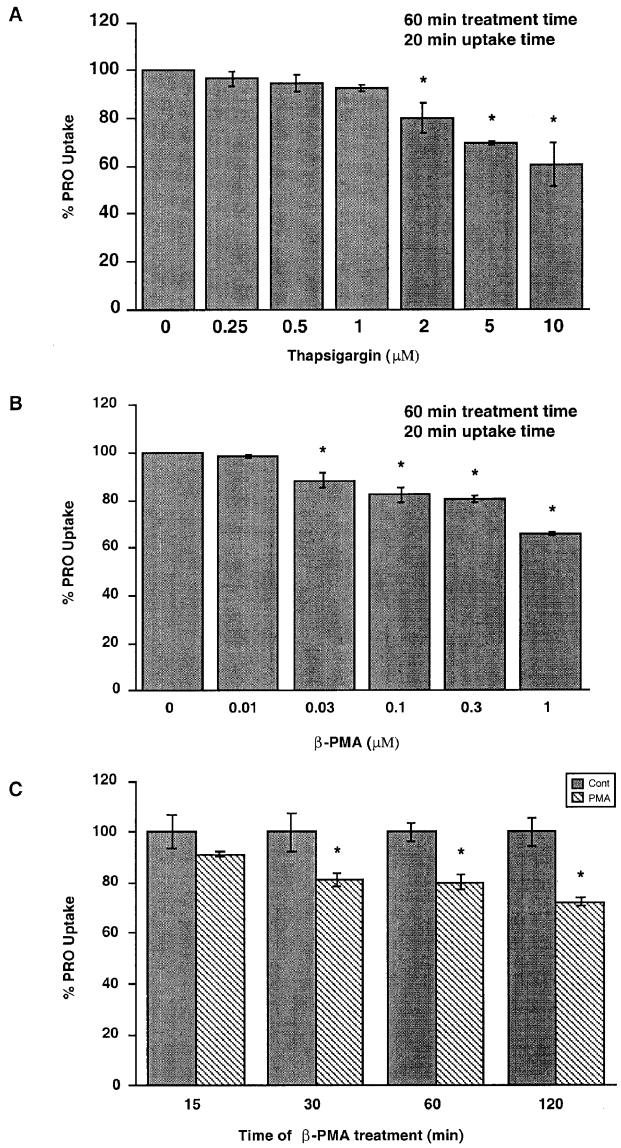

Figure 1 Thapsigargin (A) and β-PMA (B) inhibit PRO uptake in a concentration dependent manner. HP cells are incubated with 0.01–1 μM β-PMA or 0.25–10 μM thapsigargin and assayed for PRO uptake for 20 min. Data are represented as percentage of control, where control is PRO uptake in the absence of thapsigargin or β-PMA (the absolute control values from two independent experiments are 17.8±1.4 and 20.6±1.9 pmol (10^6 cells)$^{-1}$ 20 min^{-1}). HP cells were treated with β-PMA at 1 μM for increasing time periods, and assayed for PROT activity (C). Parallel assays were carried out in the presence of 100 μM GGFL to define specific PRO uptake. Data presented are mean±s.e.mean of six independent values obtained from three experiments performed in duplicate. Asterisks denote significant difference as compared to respective control PRO uptake, $P<0.05$ (Student's *t*-test and ANOVA).

spectrometry with a Microbeta microplate scintillation counter (Wallac). Radiolabelled PRO (50 nM) is used in all experiments except for kinetic studies, where unlabelled PRO was added simultaneously with radiolabelled PRO. Specific PRO uptake was measured by subtracting the PRO uptake measured in the presence of 100 μM GGFL from the total PRO uptake measured in the absence of GGFL for all our experiments. Substrate K_m and V_{max} were determined by nonlinear least-square fits (Kaleidagraph, Synergy Software) with the generalized Michaelis-Menten equation, $V = V_{max}[S]^n/(K_m{}^n + [S]^n)$, where V = transport velocity, [S] = substrate concentration, and n represents the Hill coefficient. Data are represented as mean ± s.e.mean of six independent values obtained from three separate experiments carried out in duplicates. Results presented on the effects of modulators on PRO uptake arise from experiments using vehicle treated cells, assayed in parallel. Statistical analyses were performed using both Student's *t*-test and ANOVA (INSTAT, Softengine).

Measurement of $[Ca^{2+}]_I$

$[Ca^{2+}]_I$ was determined with fura-2AM (acetoxymethyl ester) as described previously (Merrit & Rink, 1987). Briefly, HP cells seeded on fibronectin coated coverslips were incubated with 2 μM fura-2AM in KRH buffer for 30–45 min at 37°C. The cells were washed twice with KRHT buffer and incubated 20–180 min prior to recording. Fura-2 loaded cells were visualized using a Nikon inverted microscope attached to a Compix Calcium Imaging System. The imaging system consists of a Charge Coupled Device (CCD) camera (Dage-MTI CCD-72, Michigan, Indiana, U.S.A.) attached to an IBM compatible computer executing SIMCA C-Imaging software (Compix, Cranberry Township, PA, U.S.A.). $[Ca^{2+}]_I$ levels were determined by fluorescence ratio measurements using 340 nm and 380 nm excitation wavelength light supplied through interference filters. Fluorescence was continuously monitored before and after the addition of 5 μM thapsigargin and traces were stored in an Apple computer and analysed using program Igor (WaveMetrics, Inc., Lack Oswego, OR, U.S.A.).

Electrophysiological recordings

Prior to electrical recording, HP cells were plated at a density of 10^5 per 35 mm culture dish and attached cells were washed three times with bath solution at 37°C. The normal bath contained (in mM): NaCl 130, KCl 1.3, KH_2PO_4 1.3, $MgSO_4$ 0.5, $CaCl_2$ 1.5, HEPES 10, and dextrose 34 (pH 7.35 and 300 mOsm). Pipette solutions for the whole-cell recording contained (in mM): KCl 130, $CaCl_2$ 0.1, $MgCl_2$ 2, EGTA 1.1, HEPES 10, and dextrose 30 (pH 7.35 and 270 mOsm). Free Ca^{2+} in the pipette was 0.1 μM. Electrodes were pulled using a Programmable puller (Sachs-Flaming, PC-84). An Axopatch 200A amplifier band-limited at 5000 Hz was used to measure current. Data were stored digitally on VCR and analysed on a Nicolet 4094 oscilloscope and an IBM-AT computer. Statistical analyses on normalized currents were performed using both Student's *t*-test and ANOVA (INSTAT, Softengine).

Results

Thapsigargin and β-PMA modulate PRO uptake

Thapsigargin exhibited concentration-dependent inhibition on PRO uptake after exposure for 60 min (Figure 1A). We

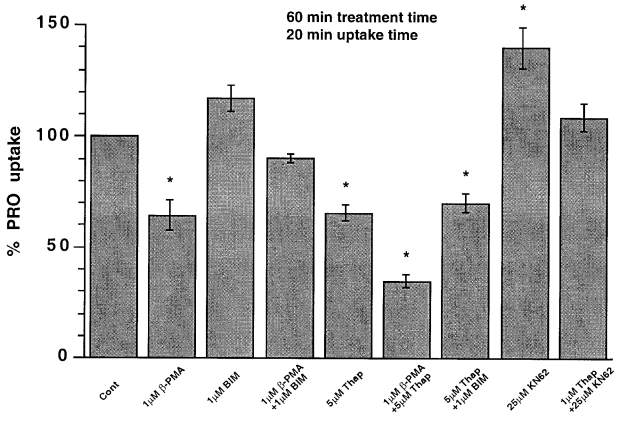

Figure 2 Thapsigargin and kinase modulators affect PRO uptake. The results of PRO uptake in HP cells (total uptake minus uptake in the presence of 100 μM GGFL) are represented as a relative percentage of the control PMA (the absolute control value from two independent experiments is 19.3 ± 2.1 pmol $(10^6 \text{ cells})^{-1}$ 20 min^{-1}). Parallel assays were carried out in the presence of 100 μM GGFL to define specific PRO uptake. Data are represented as mean ± s.e.mean of six independent values obtained from three experiments performed in duplicate. Asterisks indicate statistically significant changes as compared to control PRO uptake, $P < 0.05$ (Student's *t*-test and ANOVA).

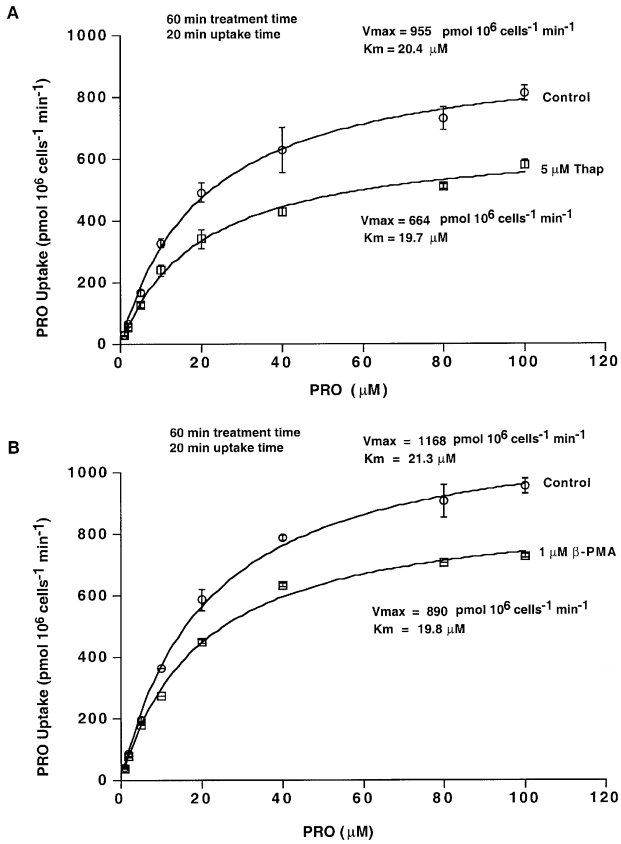

Figure 3 Thapsigargin (A) and β-PMA (B) decrease V_{max} of PROT. Experimental procedure is given under Materials and methods. HP cells were treated with and without thapsigargin in panel A or β-PMA in panel B, and PRO uptake was measured over 1–100 μM of PRO. Specific uptake represents GGFL-sensitive component. PRO uptake in pmol $(10^6 \text{ cells})^{-1}$ min^{-1} is plotted as a function of PRO concentration. Results are represented as mean ± s.e.mean of six independent values obtained from three experiments performed in duplicate. Asterisks denote significant difference in V_{max} values as compared to respective controls, $P < 0.05$ (Student's *t*-test and ANOVA).

observed 25% inhibition at 5 μM thapsigargin and used this concentration for comparisons in subsequent experiments. Similarly β-PMA showed about 35% inhibition at 1 μM (Figure 1B) after 60 min (Figure 1C), and we used this concentration and exposure for future comparisons. We first tested whether the Ca^{2+} that is released by thapsigargin activates the PKC pathway (Figure 2) by looking at PRO uptake in the presence of β-PMA or thapsigargin alone, together, or in combination with other PKC modulators. β-PMA inhibits uptake, but α-PMA (the inactive form) has no effect (data not shown). The PKC inhibitor BIM largely abolished the inhibitory effect of β-PMA. Thapsigargin inhibits uptake and cotreatment with β-PMA results in significant additive inhibition (Figure 2). BIM does not prevent the inhibitory effect of thapsigargin, suggesting that thapsigargin mediates its effect *via* signalling pathways other than PKC. KN-62, a specific CaMK II inhibitor, stimulates PRO uptake and abolishes the thapsigargin inhibitory effect suggesting that PROT is regulated by CaMK II. This is further supported by the PKC down regulation experiments, where thapsigargin, but not β-PMA, exhibited the inhibitory effect. In addition we did not observe the additive inhibition by β-PMA and thapsigargin cotreatment (data not shown).

Thapsigargin and β-PMA decrease V_{max}

The relation between uptake rate and PRO concentration was hyperbolic (Figure 3), suggesting a single saturable site for the substrate. Treatment with thapsigargin reduces V_{max} from 955 ± 30 to 664 ± 25 pmol $(10^6 \text{ cells})^{-1}$ min^{-1} with no change in K_m (from 20.4 ± 1.9 to 19.7 ± 2.2 μM) (Figure 3A). Treatment with β-PMA reduces V_{max} from 1168 ± 29 to 890 ± 28 pmol $(10^6 \text{ cells})^{-1}$ min^{-1} with no change in K_m (from 21.3 ± 1.6 to 19.8 ± 1.9 μM) (Figure 3B). The difference in control values represents normal variation between batches.

Thapsigargin affects $[Ca^{2+}]_I$ levels and PRO uptake

Although thapsigargin causes a comparatively rapid elevation of $[Ca^{2+}]_I$ (100 s), uptake is unaffected for 5 min (Figure 4). Following exposure to 5 μM thapsigargin, internal Ca^{2+}, elevates to 200 nM within 1 min (smooth traces, top time scale). However, at the peak of internal Ca^{2+}, PRO uptake is unaffected (bars, bottom time scale). Thapsigargin-induced inhibition of uptake requires 10 min to appear and persists for 60 min. These results suggest that the thapsigargin-induced inhibition of uptake illustrated in Figures 1–3 is not a direct effect of Ca^{2+} elevation but rather a modulation of uptake by down-stream Ca^{2+}-dependent pathways.

Thapsigargin increases PRO-induced currents

The effect of thapsigargin on PRO uptake may be mediated indirectly *via* the membrane potential (Galli *et al*., 1996; 1997; 1998) or may be regulated by down-stream phosphorylation events (Qian *et al*., 1997). To test whether thapsigargin affects membrane conductance, we voltage-clamped HP cells at -100 mV and monitored the current during 1 min following exposure to 5 μM thapsigargin. Thapsigargin has no effect on the holding current (data not shown), demonstrating that its effect on uptake is not *via* non-specific effects on membrane conductance. Next we tested the effect of thapsigargin on PRO-induced current. HP cells were voltage-clamped to a holding potential of -40 mV for 1 s then stepped to a test potential of -120 mV for 500 ms. Current was recorded before exposure to PRO (control), after 50 μM PRO, and 1 min after addition of 5 μM thapsigargin (Figure 5A). Thapsigargin causes an immediate 2.5 fold increase in the PRO-induced current and the PRO-induced, thapsigargin potentiated currents are specific to PROT because they are

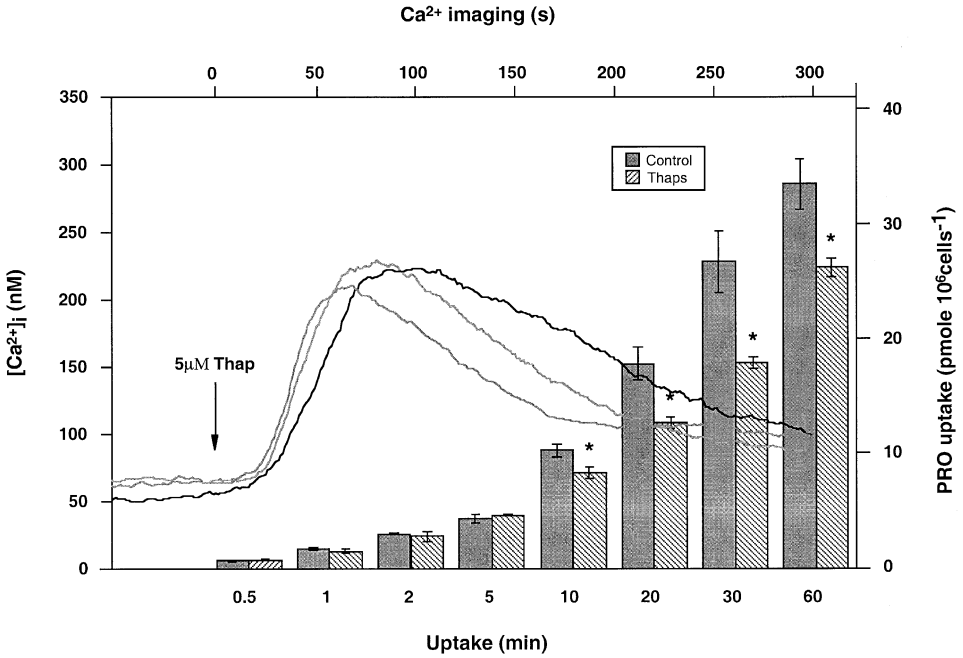

Figure 4 The effect of thapsigargin on the time course of PRO uptake and Ca^{2+} mobilization. PRO uptake was measured over a time period ranging from 30 s to 1 h at 37°C in the presence or absence of 5 μM of thapsigargin. The results are plotted in pmol $(10^6 \text{ cells})^{-1}$ min^{-1}. Also shown on a different time scale are the changes in Ca^{2+} levels (in nmoles) in three different cells after addition of 5 μM thapsigargin. Data presented are mean $\pm$ s.e.mean of six independent values obtained from three experiments performed in duplicate. Asterisks denote significant difference as compared to respective control PRO uptake, $P<0.05$ (Student's *t*-test and ANOVA).

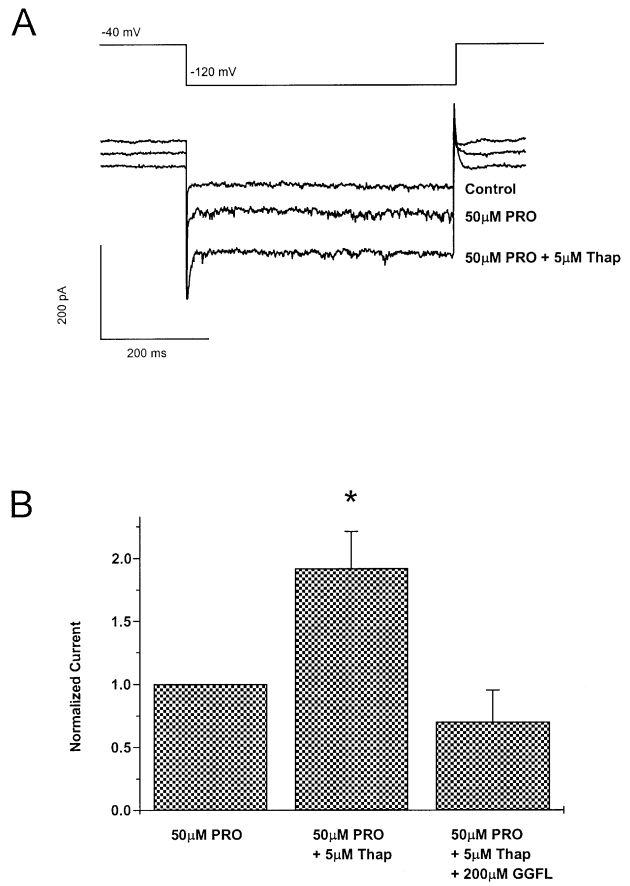

Figure 5 Thapsigargin increases PRO-induced current. Panel A shows representative traces for a single cell under three conditions: control, after addition of 50 μM PRO, and 1 min after addition of 5 μM thapsigargin along with PRO, panel B represents average values for five cells including block by GGFL of both PRO-induced and thapsigargin-potentiated PRO-currents. Asterisk denotes significant difference as compared to control PRO-induced current, $P<0.05$ (Student's *t*-test and ANOVA).

blocked by GGFL (Figure 5B). Because PRO-induced current reflects PRO uptake (Galli *et al*., 1999), the rise in $[Ca^{2+}]_I$ 1 min after thapsigargin (Figure 4) most likely potentiates uptake. However, the depolarization associated with uptake in unclamped cells (Figure 4) masks this effect, whereas in clamped cells (Figure 5) the acute effects of elevated Ca^{2+} are evident.

Discussion

It has been shown previously that Ca^{2+}-dependent protein kinases modulate PRO transport in renal brush border membrane vesicles (Zelicovic & Przekwas, 1995). This PRO transport is called Na^+-amino acid co-transport and is unrelated to the PROT we are reporting on. Here we present the first evidence for the role of Ca^{2+} and Ca^{2+}-dependent protein kinases in the regulation of high-affinity brain-specific Na^+- and Cl^--dependent PROTs. To this end we have utilized the effects of thapsigargin on PRO uptake and PRO-induced currents, specific kinase modulators, and GGFL to define PROT specific effects. The thapsigargin inhibition of PRO uptake, the effect of specific kinase modulators (Figures 1–4) and thapsigargin potentiation of PRO-induced current (Figure 5) suggest two mechanisms for PROT regulation, (1) inhibition of PROT by Ca^{2+}-dependent protein kinases and (2) potentiation of PROT directly by Ca^{2+}.

Protein kinase modulators appear to implicate PKC-dependent regulation of PROT (Figure 1). However, the additive inhibitory effect of β-PMA and thapsigargin, and the inability of BIM to prevent thapsigargin inhibition, suggest that thapsigargin inhibition is not *via* PKC-mediated pathway (Figure 2). This is supported by the ability of thapsigargin, but not β-PMA, to inhibit PROT in PKC down-regulated HP cells. A specific CaMK II inhibitor, KN-62 prevents thapsigargin inhibition (Figure 2). Thus the long-term Ca^{2+} inhibition of PROT is mediated *via* PKC and CaMK II. PKC isoforms that require no Ca^{2+} may also be involved (Nashizuka, 1992; Casabona, 1997). Chronic treatment with β-PMA actually enhanced PRO uptake (data not shown), indicating tonic regulation of PROT by PKC probably *via* trafficking or surface expression. We also observed consistent stimulation of PROT by BIM (Figure 2), which suggests tonic regulation by PKC. Similar regulation of SERT and NET by Ca^{2+}-dependent protein kinases is well established (Jayanthi *et al*., 1994; Qian *et al*., 1997; Ramamoorthy *et al*., 1995; Apparsundaram *et al*., 1998a,b). Although we have no direct evidence to support regulation of surface expression, our data are the first to show that protein kinases can regulate the brain-specific, high-affinity PROT.

Although thapsigargin alone has no effect on membrane voltage, it immediately increased the PRO-induced current (Figure 5) coincident with the expected elevation in $[Ca^{2+}]_I$ (Figure 4). Because PRO-induced current directly reflects PRO transport (Galli *et al*., 1999) we conclude that, prior to its down-stream inhibition of PROT, calcium acts as an acute activator of PROT. Demonstration of this initial calcium activation of PRO uptake relies on voltage control, because depolarization that accompanies PRO uptake masks the effect. Depolarization is not a likely explanation for long-term inhibition of uptake, because cells are only transiently depolarized.

Calcium regulation of PRO uptake may be prominent, because PROTs are present in sub-populations of putative glutamatergic neurons (Fremeau *et al*., 1992; Nadler *et al*., 1992; Velaz-Faircloth *et al*., 1995), suggesting a role for PRO and PROT in excitatory neurotransmission. This hypothesis is supported by the presence of PROTs in small synaptic vesicles (SSVs) within terminals forming asymmetric excitatory-type synapses in the CPN and CA1 regions of hippocampus (Renick *et al*., 1999). PROTs could modulate excitatory transmission by altering the concentration of PRO, the presumed natural substrate. Regardless of these interpretations, the demonstration of acute activation of a neurotransmitter transporter suggests that calcium is critical not only for the release of transmitter, but also to its timely removal from the synaptic cleft, whereas slower down-stream inactivation may be important for long-term modulation of synaptic transmission.

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