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Genistein Inhibits Na^+/Ca^{2+} Exchange Activity in Primary Rat Cortical Neuron Culture

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We have examined the possible regulatory effect of tyrosine kinase activity on Ca^{2+} transport observed in the cultured rat cortical neurons. Na^+/Ca^{2+} exchange was studied using cells cultured for various time periods. A nearly two fold increase in Ca^{2+} uptake was seen when comparing 3 day and 9 day cultures. Western blot analysis also showed a two fold increase in Na^+/Ca^{2+} exchanger (NCX1) protein levels as cells matured in culture. To study the effect of genistein (a specific tyrosine kinase inhibitor) cells were incubated with 100μM genistein (in 1% DMSO) for 1 hour before the assay of Na^+/Ca^{2+} exchange activity. There was a significant decrease of Ca^{2+} uptake in genistein treated neurons (control: 4.596±0.205 nmol/mg protein/15min, n=12; genistein: 1.420±0.131 nmol/mg protein/15min, n=12, mean±S.E. P<0.001). Daidzein, an inactive analog of genistein and phorbol myristate acetate (PMA), a PKC activator were without effect. The results suggest that as cells mature in culture, Na^+/Ca^{2+} exchange capacity increases, as a result of greater protein expression. Exposure to genistein inhibited Ca^{2+} uptake suggesting that the exchanger may be modulated by tyrosine phosphorylation.

The Na^+/Ca^{2+} exchanger is a plasma membrane antiporter that is thought to play a primary role in calcium homeostasis in neurons. At present, three isoforms, NCX I (1), NCX II (2) and NCX III (3) have been cloned and all three are expressed in the rat brain. Experimental evidence suggests that the large intracellular loop between transmembrane segments 5 and 6 contains the sites that are involved in the regulation of Ca^{2+} transport (3,4). Near the amino-terminal end of the intracellular loop, there is a 20 amino acid portion which has the same sequence as the exchange inhibitory protein (XIP). The application of XIP to the intracellular side of the membrane reduces Ca^{2+} transport. In addition, β-1 and β-2 repeats, which are contained in the intracellular loop, have Ca^{2+} binding sites that are distinct from the Ca^{2+} transport site. Recent work in transfected CHO cells suggests that Ca^{2+} released from intracellular stores binds to these sites and activates Ca^{2+} transport (5).

Early experiments suggested regulation of Na^+/Ca^{2+} exchange activity by a phosphorylation/dephosphorylation process (6) in cardiac muscle. However, those results were never reproduced by others. More recent experimental evidence has suggested regulation of Na^+/Ca^{2+} exchange activity by Ca^{2+}/calmodulin-dependent protein kinase (7) and protein kinase C (8,9). The deduced amino acid sequences of exchanger isoforms expressed in brain reveals a potential tyrosine kinase phosphorylation site in each isoform. A report has appeared in abstract form of the inhibition of Na^+/Ca^{2+} exchange activity stably expressed in CHO cells by genistein, an inhibitor of tyrosine kinase (10). In this study, we have examined the effect of genistein on calcium uptake mediated by Na^+/Ca^{2+} exchange in cultured rat cortical neurons.

METHODS

Rat cortical culture. Primary culture of embryonic cortical neurons was performed as described by Mattson et al. (11). Six-well culture plates (Falcon) were coated with polyethyleneimine (50% solution, Sigma, St. Louis, MO, USA), which was diluted 1:1000 in borate buffer. Rat cortical neurons from E-18 rats were dissociated and plated on the coated culture plates. The cortical cells were cultured at 37°C, 5% CO_2 in MEM solution (Gibco, Grand Island, NY, USA) supplemented with 10mM sodium bicarbonate, 2mM L-glutamate, 1mM pyruvate, 20mM KCl and 10% (v/v) fetal bovine serum (Gibco). After 5 day of culture, 10μM of Ara-C (Sigma) was added to control the proliferation of glial cells.

Exchange activity. Na^+/Ca^{2+} exchange activity was assayed as described by Vemuri et al. (12) with slight change. Neurons were sodium loaded in buffer containing 132 mM NaCl, 1 mM ouabain, 25 μM nystatin, 2mM $MgCl_2$, and 10 mM HEPES on ice for 10 minutes. Na^+/Ca^{2+} exchange was initiated by incubating the neurons at 37°C for 15 minutes with either 137 mM NaCl, 0.1 mM EGTA, 0.55 mM $CaCO_3$, 1 mM ouabain, 10 mM HEPES and 0.83μCi/ml $^{45}Ca^{2+}$ (buffer A) or the same buffer with 137 mM choline chloride instead of 137 mM NaCl (buffer B). The free calcium concentration in the above solutions was 257μM verified using a calcium sensitive elec-

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trode (13). The exchange was terminated by addition of 500mM $LaCl_3$ in HEPES buffer. The neurons were washed with La^{3+} three times, then lysed with NaOH and lysate radioactivity was determined by liquid scintillation counting. Protein concentration for each sample was determined by Lowry's method (14). Na^+-dependent Ca^{2+} uptake was measured by subtracting the values obtained in buffer B from the values obtained in buffer A. To detect the effect of K^+ and Ni^{2+} on the Na^+/Ca^{2+} exchange, 10 mM K^+ or 5 mM Ni^{2+} were added to both buffer A and buffer B.

Effect of genistein, daidzein and phobol myristate acetate (PMA). Genistein and its inactive analog, daidzein were obtained from Research Biochemical International (RBI, Natick, MA), and PMA was from Sigma (St. Louis, MO) These reagents were dissolved in dimethylsufoxide (DMSO), prepared in aliquots, and frozen till use. To determine the effect of genistein, cells were pre-incubated with 1% DMSO, 100μM daidzein in 1% DMSO, 100μM genistein in 1% DMSO or 100ng/ml PMA for one hour before the exchange activity assay. Genistein, daidzein, or PMA were contained in the Na^+ loading buffer, and buffer A and B described above.

Preparation of plasma membrane fraction from cultured cortical cells. Plasma membranes were purified as described elsewhere (15) with slight modification. The medium was removed and cultured cells were stored frozen at −70°C until use. The cells in a 60-mm culture dish were scraped off in 1ml solution containing 0.32 mM sucrose, 10 mM HEPES, pH 7.4 and protease inhibitors (soybean trypsin inhibitors, 50 μg/ml; pepstatin, 0.7 μg/ml; and leupeptin, 0.5 μg/ml) and transferred to a glass tissue grinder (Pyrex No. 7250). The dish was rinsed with 2ml of the above solution again and the buffer was transferred to the same grinder. The tissue was homogenized by 20 up-down strokes in the grinder. The homogenate was centrifuged at 1,000×g for 10 minutes and supernatant was transferred to a centrifuge tube. The pellet was re-suspended and centrifuged again as above and supernatant was pooled in the same tube. The pooled supernatant was centrifuged at 5,000×g for 5 minutes to pellet nuclei and mitochondria. The supernatant was then centrifuged at 125,000×g for 45 minutes to sediment the plasma membrane fraction. The fractions were re-suspended in 0.32M sucrose and stored at −70°C until use.

Western blot. The Western blot analysis was performed as described previously (16). Samples were boiled in buffer containing 10 mM NEM. The proteins were transferred to a nitrocellulose membrane. The membrane was incubated with affinity purified anti-peptide antibody to the NCX I isoform and the blot was developed by using the Renaissance chemiluminescent kit (DuPont). The blot was exposed to the film for various periods of time and the film was scanned and analyzed by a computer driven densitometer (Bio-Rad GS670).

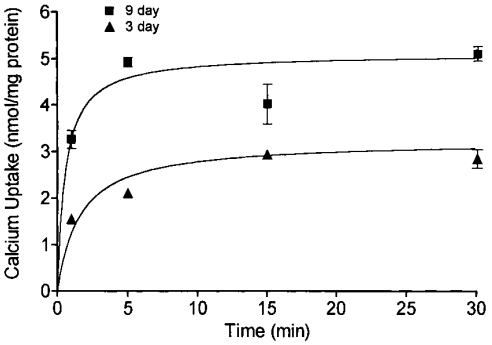

FIG. 1. Time course of changes of Na^+-dependent calcium uptake by rat cortical cells in culture. Ca^{2+} uptake was determined as described in Methods. Each point represents the mean value ± S.E. (n=3). Day 3 and Day 9 culture give two different curves as indicated by two-way ANOVA (F=197.79, P<0.0001).

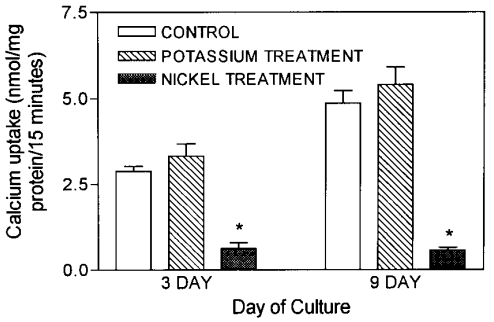

FIG. 2. Effect of 10mM K^+ and 5mM Ni^{2+} added extracellularly on Na^+-dependent Ca^{2+} uptake in 3 day and 9 day cultures. Ca^{2+} uptake was assayed 15 minutes after initiation of an exchange reaction as described in figure 1. "*" indicates that there was a significant difference between control and nickel treatment (t=11.17, P<0.0005).

RESULTS AND DISCUSSION

After the neurons were loaded with Na^+, Ca^{2+} transport was measured for 1, 5, 15 and 30 minutes to determine the time course Ca^{2+} uptake. Figure 1 shows the time course experiments for both day 3 and day 9 cultures. At 1 minute, the exchange activity was the lowest (3 day: 1.56±0.10, n=3; 9 day: 3.26±0.19, n=3; mean±SE), then it increased significantly at 5 minutes (3 day: 2.11±0.09, n=3; 9 day: 4.93±0.10, n=3, mean±SE) and gradually reached a plateau level at 15 minutes. Therefore, a 15 minute incubation with exchange buffer was sufficient to yield maximal Ca^{2+} uptake values in determining the exchange activity for the following experiments. Two-way ANOVA indicated that day 3 and day 9 culture give two significantly different curves. Thus, the 9 day culture gave higher Na^+-dependent calcium uptake compared with the 3 day culture.

The observed Na^+-dependent Ca^{2+} uptake by cortical neurons could be mediated by influx pathways other than the Na^+/Ca^{2+} exchanger. To confirm that Ca^{2+} uptake was mediated by the Na^+/Ca^{2+} exchanger, a specific and effective inhibitor is needed. XIP is a specific inhibitor, but only effective when it is applied intracellularly, because XIP itself can not cross plasma membrane. Amiloride derivatives can inhibit Na^+/Ca^{2+} exchange when applied extracellularly, but are relatively nonspecific. It has been reported that Ni^{2+} blocks the Na^+/Ca^{2+} exchange current in ventricular cells from guinea-pig hearts and has little effect on the Ca^{2+}-Ca^{2+} exchange current (17). In figure 2, neurons were treated with 5mM Ni^{2+} and it was found that Na^+ dependent Ca^{2+} uptake was almost completely abolished. The inhibitory effect reduced the Na^+ dependent Ca^{2+} uptake in cortical culture to around 0.5-0.7 nmol/mg

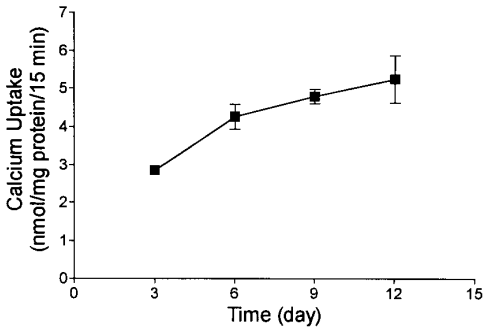

FIG. 3. Maximal calcium uptake observed in rat cortical cells in culture for different lengths of culture period. At 3, 6, 9, and 12 days after initial plating, calcium uptake was assayed as described in figure 2. Each point represents mean value ± S.E. (n=15 for day 3 and day 9 cultures, n=11 for day 6 and day 12 cultures). Significant increase in calcium uptake was between day 3 and day 6, as indicated by one one-way ANOVA ($F=11.66$, $P<0.0001$).

protein/15 min regardless of how long the cortical cells were cultured. The inhibition by nickel suggests that the Na^+ dependent Ca^{2+} uptake observed in this study most likely is mediated by the Na^+/Ca^{2+} exchanger. It should be noted that Ni^{2+} is not a specific Na^+/Ca^{2+} exchanger inhibitor, it may interact with voltage sensitive or receptor operated calcium channels. Further experiments using calcium channel antagonists will provide additional evidence for Ca^{2+} channel involvement in Ca^{2+} uptake, if any.

Two studies, one in squid giant axon (18) and the other one in rat brain synaptic plasma membrane (SPM) vesicles (19), showed that K^+ had stimulatory effects on the Na^+/Ca^{2+} exchanger. Allan and Baker (20) suggested that in squid giant axon, K^+ acted directly on the Na^+/Ca^{2+} exchanger instead of modulating Na^+/Ca^{2+} by changing the membrane potential. Using rat brain SPM, studies with K^+ suggested transport of K^+ as well. Thus, we were interested in determining the effect of K^+ on Na^+/Ca^{2+} exchange in primary cortical culture. As shown in figure 2, Na^+ dependent Ca^{2+} uptake showed a trend toward increased values in the presence of extracellular K^+, however the increases were not significant.

Figure 3 shows maximal Na^+-dependent Ca^{2+} uptake of neurons cultured for 3, 6, 9, and 12 days. A significant increase was seen between day 3 (2.86 ± 0.11, n=15) and day 6 (2.26 ± 0.33, n=11) of culture, as indicated by one-way ANOVA. The maximal exchange activity reached a plateau level around 9 days of culture. The exchange activity approximately doubled by day 12 of (5.24 ± 0.63, n=11) culture when compared with day 3 of culture. This activity elevation paralleled significant morphological changes in the neurons during this period. Most neurons had shorter and fewer neurites at the third day of culture when compared to the neurons cultured for 9 days. The neurons from 9 day culture also had more synapse formation. The neuronal maturation and its accompanying elevation of Na^+/Ca^{2+} exchange activity were quite similar to the results from Johaszova et al. (21). In that experiment, a rise of Na^+/Ca^{2+} exchange activity during the ontogeny of rat brain was found between postnatal day 2 and day 20. Since intracellular calcium elevation is important for proper brain development, such as neural induction (22), growth cone mobility (23), neurotransmitter release (24), neuronal excitability (25) and neurite elongation (26); a proportional increase in the activity of the Na^+/Ca^{2+} exchanger to prevent neuronal calcium overload is not surprising.

The mechanism underlying the increase in Na^+/Ca^{2+} exchange activity as neurons matured in culture was due to increased exchange protein (NCX I) expression, as shown by Western analysis (Figure 4). The antibody used was quite specific to the protein product of the NCX I gene (27). Panel A shows that a 122 kDa protein was labeled in both day 3 and day 9 cultures. It was clear that day 9 cultures had a higher amount of Na^+/Ca^{2+} exchanger protein than day 3 cultures. Densitometric analysis (panel B), based on 8 samples from either 3 day or 9 day cultures, indicated that the amount of Na^+/Ca^{2+} exchanger protein (NCX I) in 9 day cultures (1.51 ± 0.37, n=8) was doubled when compared to 3 day cultures (4.18 ± 0.91, n=8). The increase in NCX I protein paralleled the increase in exchange activity. The Na^+/Ca^{2+} exchanger is not the only Ca^{2+}

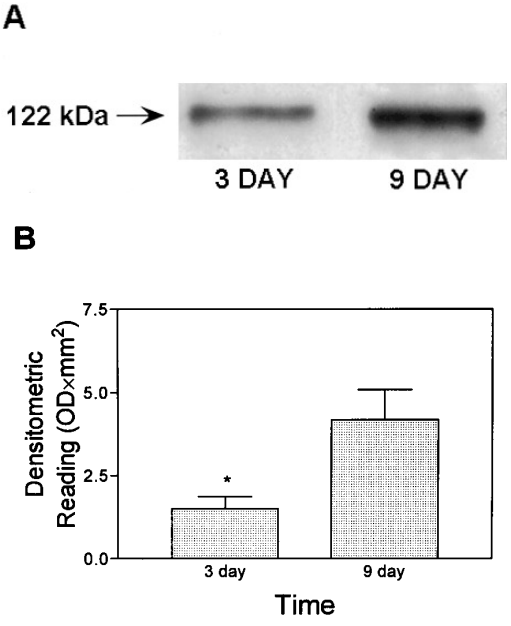

FIG. 4. (A) Western blot analysis of the Na^+/Ca^{2+} exchanger (NCX I) protein expression. Each lane represents 50 μg of plasma membrane protein isolated from cortical cultures of 3 or 9 days. (B) Each bar represents mean values ± S.E. obtained from densitometric analysis of Western blots (n=8). "*" indicates that the difference between day 3 and day 9 cultures was significant. ($t=2.744$, $P<0.05$).

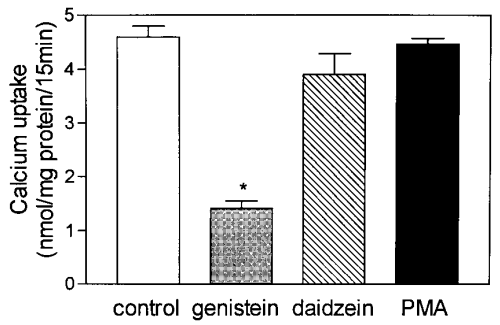

FIG. 5. The effect of genistein treatment on maximal Na^+-dependent Ca^{2+} uptake. For control cells, DMSO was added to culture media to obtain a final concentration of 1%. Genistein (final concentration is 100μM) or daidzein (100μM) was added to the media (DMSO final concentration was 1%). PMA (final concentration was 100ng/ml) was added without DMSO. The cells were incubated in each media for 1 hour before Na^+ loading. Then Ca^{2+} uptake was initiated as described in the methods. To both buffer A and buffer B, 1% DMSO, or 100μM genistein, or 100μM daidzein or 100ng/ml PMA was added. "*" indicates significant difference between control and genistein treatment. ($q=12.36$, $P<0.001$).

homeostatic protein that increases in maturing neurons. Calbindin-D, an intracellular calcium binding protein, was found to increase gradually in cultured rat hippocampal neurons from culture day 3 to culture day 10 (28). Further study by Northern blot of mRNAs will elucidate if this elevation of exchanger protein is induced by increased mRNA transcription.

The neurotrophins, such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT 4/5) are important for neuron survival and its normal function in central nervous system (CNS). All of them are reported to bind and activate a family of receptor protein tyrosine kinases (PTK)---Trk (29, 30, 31), which in turn increase intracellular Ca^{2+} concentration (32). This may contribute to the local protein synthesis in neurotrophin-induced synaptic plasticity (33). It is also suggested that protein tyrosine kinase phosphorylation is involved in the activation of calcium channels in different tissues (34, 35, 36), which results in increased calcium influx. Based on these findings, we propose the hypothesis that protein tyrosine kinase activity may also simultaneously regulate the Na^+/Ca^{2+} exchanger directly or indirectly to maintain intracellular calcium homeostasis. It has been reported that genistein, a tyrosine kinase inhibitor, inhibited Na^+/Ca^{2+} exchange in NCX II cDNA transfected CHO cells (10). In figure 5, it is shown that pre-incubation of cortical neurons for 1 hour with 100μM genistein resulted in around 70% inhibition of Na^+ dependent Ca^{2+} uptake. The calcium uptake of the control was 4.596 ± 0.205, $n=12$, and that of genistein treated neurons was 1.420 ± 0.131, $n=12$. Daidzein, the inactive analog of genistein, did not have a significant effect on Ca^{2+} uptake. Phobol myristate acetate (PMA), the PKC activator, also had no effect. Additional experiments extended the incubation time with genistein to 24 hours and increased the concentration to 200μM, but no further inhibition was observed (data not shown). These data suggest that the brain Na^+/Ca^{2+} exchanger isoforms can be regulated by tyrosine kinase phosphorylation but not by PKC phosphorylation.

Important differences exist between the present findings and those reported previously by Reeves (10). In that study, genistein reduced exchanger activity by maximally 75% in Chinese hamster ovary (CHO) cells transfected with NCX II cDNA, but not in CHO cells transfected with NCX I cDNA. In our experiment, the parallel increases in NCX I protein expression and exchange activity during the culture period indicated that NCX I was the primary isoform responsible for Ca^{2+} transport in cultured cortical neurons. The inhibition of Ca^{2+} transport by genistein suggested that NCX I can be modulated by tyrosine kinase phosphorylation in neurons. However, it can not be excluded that the other two isoforms, NCX II and NCX III are also modulated by tyrosine kinase phosphorylation. Sequence analysis indicates the Tyr-581 in NCX I, Tyr-601 in NCX-II and Tyr-608 in NCX III are potential tyrosine kinase phosphorylation sites (3).

There are at least two possible models to explain modulation of the Na^+/Ca^{2+} exchanger by tyrosine kinase activity. One model postulates that either the exchanger itself is phosphorylated and this in turn results in activation, or the binding affinity of a regulatory protein (cytosolic protein) is increased by tyrosine phosphorylation. Phosphorylation of either the exchanger or the regulatory protein or both could affect binding affinity. As yet, no experimental evidence exists that the exchanger is actually tyrosine phosphorylated or for the existence of a cytosolic regulatory protein. A second model postulates that activation of protein tyrosine kinase receptors (e.g. NGF receptor, BDNF receptor, etc.) results in release of Ca^{2+} from intracellular stores (via phospholipase C-γ activation and release of inositol triphosphate). The resulting elevation in intracellular Ca^{2+} then activates Na^+/Ca^{2+} exchange via Ca^{2+} binding sites on the large intracellular loop. This pathway, when interrupted by genistein, results in lowered intracellular Ca^{2+} and reduced Na^+/Ca^{2+} exchange activity. In support of this model, thapsigargin treated CHO cells expressing the bovine cardiac Na^+/Ca^{2+} exchanger exhibit accelerated Ca^{2+} transport (5).

The lack of an effect of pre-incubation with PMA deserves some discussion. It has been reported that phobol 12,13-dibutyrate, a PKC activator stimulated Na^+/Ca^{2+} exchange in rabbit abdominal aorta (8), but in a renal epithelial cell line, activation of PKC strongly depressed Na^+/Ca^{2+} exchanger expression (37). Although potential phosphorylation sites for PKC exist

in the exchanger sequence, PKC did not show any significant effect on Na^+/Ca^{2+} exchange in our experiments. These data may reflect that PKC modulation of the Na^+/Ca^{2+} exchanger is tissue specific.

In summary, the present study shows that Na^+/Ca^{2+} exchanger activity increases during maturation of neurons in culture for as long as 12 days. This change was most likely due to the elevated expression of Na^+/Ca^{2+} exchanger protein. The exchange activity was inhibited by Ni^{2+}, but no significant effect was found by increasing extracellular K^+. Genistein, a tyrosine kinase inhibitor, partially inhibited the Na^+-dependent Ca^{2+} uptake. The mechanism by which inhibition of tyrosine kinase activity is linked to Na^+/Ca^{2+} exchange activity has yet to be elucidated.

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