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Research Report

Reverse mode Na^+/Ca^{2+} exchangers trigger the release of Ca^{2+} from intracellular Ca^{2+} stores in cultured rat embryonic cortical neurons

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ABSTRACT

The importance of Na^+/Ca^{2+} exchangers in the regulation of the physiological and pathological functions of the nervous system has been widely recognized. In this study, we used primary cultured E14.5 cortical neurons as a model system to study the possible roles of the reverse mode Na^+/Ca^{2+} exchange activity in neurotransmission. Using RT-PCR, several exchanger isoforms, *ncx1*, *ncx3* and *nckx2–4* were found to be expressed in freshly isolated and cultured cortical neurons. Expression of *ncx2* was undetectable in freshly isolated neurons but increased with time in culture. Neurons were treated with ouabain to increase the intracellular Na^+ concentration and the extracellular Na^+ was replaced by *N*-methyl-D-glucamine to activate reverse mode Na^+/Ca^{2+} exchange. During the maturation of the neurons, the exchange activity shifted from mostly K^+-dependent exchange to both K^+-dependent and K^+-independent exchange. The $[Ca^{2+}]_i$ rises were mostly suppressed by ryanodine and thapsigargin treatments, indicating contributions from the intracellular Ca^{2+} stores. This $[Ca^{2+}]_i$ elevation could propagate to the axon terminal and resulted in elevated $[Ca^{2+}]_i$ at the postsynaptic neurons based on the fact that the elevation in the postsynaptic neuron was inhibited by 6-cyano-7-nitroquinoxaline-2,3-dione and tetanus toxin. When neurons were stimulated by AMPA to increase the intracellular Na^+ concentration, the $[Ca^{2+}]_i$ elevations were significantly inhibited by thapsigargin pretreatment and by KB-R7943. These results demonstrate that, in cultured cortical neurons, the influx of Na^+ through the ionotropic glutamate receptor activates reverse Na^+/Ca^{2+} exchange, which then triggers the release of Ca^{2+} from intracellular Ca^{2+} stores to enhance Ca^{2+} signaling and neurotransmitter release.

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Abbreviations: AMPA, α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid; AP5, D-2-amino-5-phosphonopentanoate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CPA, cyclopiazonic acid; D.I.V., days *in vitro*; NCKX, K^+-dependent Na^+/Ca^{2+} exchanger; NCX, K^+-independent Na^+/Ca^{2+} exchanger; rNC(K)X, reverse mode Na^+/Ca^{2+} exchange; NMG, *N*-methyl-D-glucamine; Ry, ryanodine; RyR, ryanodine receptor; TBOA, DL-threo-β-benzyloxyaspartate; Tg, thapsigargin; TTX, tetrodotoxin

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1. Introduction

A change in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is a prerequisite of many cellular physiological activities. Therefore, precise spatial and temporal control of Ca^{2+} is an important issue for Ca^{2+} signaling. Intracellular organelles, including the endoplasmic reticulum (ER), mitochondria and secretory vesicles, have Ca^{2+} uptake mechanisms to remove Ca^{2+} from the cytosol and bring the $[Ca^{2+}]_i$ back to the resting level. In the plasma membrane, the Ca^{2+} pump and the Na^+/Ca^{2+} exchangers are the two main pathways for exporting Ca^{2+} out of the cell. Using these mechanisms, the $[Ca^{2+}]$ in various regions of the cell may be differentially modulated (Rizzuto and Pozzan, 2006; Schneggenburger and Neher, 2005; Verkhratsky, 2005).

The Na^+/Ca^{2+} exchangers play a dominant role in removing Ca^{2+} from the cytosol in cells that require extracellular Ca^{2+} for their physiological activities, such as neurons and cardiac muscle cells. Two families of Na^+/Ca^{2+} exchangers, the K^+-independent (NCX) and the K^+-dependent (NCKX), have been identified. Both exchangers are encoded by multigene families; three genes, *ncx1*, *ncx2* and *ncx3*, code for the NCX, (Canitano et al., 2002; Linck et al., 1998) and at least four genes, *nckx1*, *nckx2*, *nckx3* and *nckx4* code for the NCKX (Kiedrowski et al., 2002; Kip et al., 2006). NCX is found in almost every type of cell; while NCKX1 was originally identified in retinal cells and NCKX2–4 were later found to be expressed in the brain. It has been shown that the expression profiles of these isoforms change during brain development (Lytton et al., 2002; Papa et al., 2003).

The exchangers act in a reversible and electrogenic way with a stoichiometry of 3 Na^+ in exchange for 1 Ca^{2+} for the NCX system and 4 Na^+ in exchange for 1 Ca^{2+} and 1 K^+ for the NCKX system (Blaustein and Lederer, 1999). The reversal potential of the exchangers under physiological conditions is about +100 mV, much more positive than the resting potential. Therefore, the exchangers usually work in the forward mode to remove Ca^{2+} from the cell. It is possible that on stimulation, because the opening of the ionotropic receptors and membrane depolarization can cause the collapse of the cell Na^+ and K^+ gradients, there is an induction of the exchangers to function in the reverse mode (rNC(K)X) in order to bring Ca^{2+} into the cell (Schnetkamp, 2004). It has been shown that Ca^{2+} influx through the rNCX in trout atrial cardiomyocyte elicits Ca^{2+} release from sarcoplasmic reticulum and activates contraction (Hove-Madsen et al., 2003). However, the functions of this Ca^{2+} influx elicited by reverse mode exchange activity in modulating Ca^{2+} signaling in neurons are not very clear.

The importance of Na^+/Ca^{2+} exchangers in the regulation of the physiological and pathological functions of the central nervous system has been widely recognized. In this study, we used primary cultured embryonic cortical neurons as a model system to study the expression profile of the two gene families *ncx* and *nckx*. Furthermore, we explored the possible roles of reverse mode exchange in neurotransmission. Our results show that expression of the *ncx* and *nckx* isoforms changes with the time that the cortical neurons have been in culture. Further, we found that ionotropic α-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) receptor-induced Na^+ influx led to the activation of rNC(K)X systems and triggered the release of Ca^{2+} from intracellular Ca^{2+} stores to enhance $[Ca^{2+}]_i$ elevation and subsequently glutamate release at the axon terminal. These results suggest that, in addition to electric action potential firing, the activation of the rNC(K)X systems provides another pathway whereby there is triggering of neurotransmitter release, which then activates the postsynaptic neurons. For neurons in a network structure, the Na^+/Ca^{2+} exchangers may modulate neurotransmission by shaping the Ca^{2+} responses.

2. Results

2.1. *NCX and NCKX are differentially expressed in cultured cortical neurons*

To examine the expression of the various *ncx* and *nckx* isoforms in cultured E14.5 cortical neurons, specific primer sets (Table 1) against each isoform were used to amplify cDNA from the mRNA isolated from freshly isolated cortical neurons or neurons at 4, 6, 8 and 14 D.I.V (days *in vitro*)(Fig. 1). The results showed that *ncx1*, *ncx3*, *nckx2*, *nckx3* and *nckx4* genes were constitutively expressed in freshly isolated and cultured cortical

Table 1 – Primers for RT-PCR

Gene	Primer[1]	GI number	Expected size (bp)
NCX1	F: GGGAGGACTTTGAGGACACCTG	78214330	461
	R: GAGGGCCAGGTTCGTCTTCTTA	288229	352
NCX 2	F: TGGTGGTGTGCACTACGAGGAT	17530966	382
	R: AAACTGCTCCCTCCATGAGTGG		
NCX 3	F: ACAGTAGAAGGAACAGCCAAGGGT	17530968	435
	R: TCCTGCTGCACTAACAGTGATGG		
NCKX1	F: TCTGTCCTTGGAATGGCCTGA	9910551	265
	R: AATTGAAGTGCCTGCCGCTAG		
NCKX2	F: ATCTTGGCAGCTGGAACCTCTATC	13994178	313
	R: ACGCGAAGTAGAGGCCAAACAT		
NCKX3	F: CCAGCCTCATTGTAGCCAGACA	17160891	370
	R: GCAGGTGACCTGGTCAATCATTAG		
NCKX4	F: TGGCAGTCTCTAACACCATCGG	26024350	428
	R: AAGAGTTGACAGTGGCTGCCAA		

Primers are in 5′ to 3′ direction; F indicates the forward primer and R indicates the reverse primer.