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Striatal *proenkephalin* gene induction: coordinated regulation by cyclic AMP and calcium pathways

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Abstract

Enkephalin modulates striatal function, thereby affecting motor performance and addictive behaviors. The *proenkephalin* gene is also used as a model to study cyclic AMP-mediated gene expression in striatal neurons. The second messenger pathway leading to *proenkephalin* expression demonstrates how cyclic AMP pathways are synchronized with depolarization. We show that cyclic AMP-mediated regulation of the *proenkephalin* gene is dependent on the activity of L-type Ca^{2+} channels. Inhibition of L-type Ca^{2+} channels blocks forskolin-mediated induction of *proenkephalin*. The Ca^{2+}-activated kinase, Ca^{2+}/calmodulin kinase, as well as the cyclic AMP-activated kinase, protein kinase A (PKA), are both necessary for the induction of the *proenkephalin* promoter. Similarly, both kinases are needed for the L-type Ca^{2+} channel-mediated induction of *proenkephalin*. This synchronization of second messenger pathways provides a coincidence mechanism that gates *proenkephalin* synthesis in striatal neurons, ensuring that levels are increased only in the presence of activated PKA and depolarization.

Keywords

Enkephalin; Protein kinase A; Ca^{2+}/calmodulin kinase; L-type Ca^{2+} channels; Depolarization; Striatum

1. Introduction

The neuropeptide enkephalin plays a role in biological processes as diverse as nociception [21], stress- and immune response [7,23] and reproductive function [8]. In the central nervous system, the striatum (caudate, putamen, nucleus accumbens) is among the brain areas with the highest levels of enkephalin, which is mostly expressed in striatopallidal neurons [10,24]. Upon release, enkephalin interacts preferentially with delta and mu opiate receptors [22] and exerts a negative feedback mechanism to regulate the responsiveness of striatopallidal neurons [24]. Striatal enkephalin has been associated with motor behaviors [1,3] as well as addictive mechanisms such as reward [11,19]. Enkephalin release is precipitated by Ca^{2+}-dependent depolarization [13] and pools are replenished via induction

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of *proenkephalin* mRNA synthesis [4,6]. Two adjacent DNA enhancer elements within 110 bp 5′ to the mRNA cap site act synergistically to activate *proenkephalin* mRNA synthesis [4,6]. These enhancer elements are responsive to cyclic AMP and Ca^{2+} stimulated second messenger pathways. Here we show that L-type Ca^{2+} channels mediate the induction of mRNA synthesis of the *proenkephalin* gene for both pathways.

2. Materials and methods

2.1. Materials

FPL 64176, forskolin and nifedipine were obtained from Sigma (St. Louis, MO, USA). KN62 (1-[*N*,*O*-bis-(5-iso-quinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine) and H89-dihydrochloride (*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide) were obtained from Calbiochem (La Jolla, CA, USA).

2.2. Methods

E18 Sprague–Dawley rat pups were used for all experiments. Primary striatal cultures were obtained as previously described [18]. The *pENKAT12*, Δ80 and −772 constructs were obtained from Michael Comb. The *pENKAT12* construct contains the minimal 5′ enhancer of the human *proenkephalin* gene, 193 bp 5′ of the CAP site. The Δ80 construct contains the 84 bp 5′ of the CAP site and is not responsive to forskolin in cell culture [4,6], and the −772 construct contains the 772 bp 5′ of the CAP site. All constructs were fused to the reporter gene chloramphenicol acetyltransferase within exon 2, and contain 1.2 kb of 3′ flanking sequence of the human *proenkephalin* gene [4,6]. These constructs have been used previously in primary striatal culture by our group [14]. No differences in gene regulation pattern have been observed between *pENKAT12* and longer constructs, however, *pENKAT12* was most responsive [14]. Enkephalin constructs were transfected into primary striatal cultures using Ca^{2+} transfection [25], as described previously [18]. RNA was extracted and analyzed as described previously [18].

Kinase inhibitors were added 30 min before the addition of forskolin or FPL 64176, and were present during the exposure to forskolin or FPL 64176. Exposure to forskolin or FPL 64176 lasted 6 h.

2.3. Determination of kinase inhibitor concentration

The concentrations of KN62 and H89 needed for reliable inhibition were determined in preliminary studies using CREB phosphorylation as the model. The transcription factor CREB has been shown to be involved in forskolin-mediated induction of the *proenkephalin* gene [14,15]. The inhibitory potential of KN62 on CREB phosphorylation by glutamate, NMDA, KCl and FPL 64176 was determined in dose–response curves. A concentration of 30 μM KN62 inhibited CREB phosphorylation by NMDA and KCl, and significantly attenuated CREB phosphorylation by glutamate and FPL 64176, and was therefore chosen for the study. H89 was tested in dose–response curves with forskolin and dopamine, and 20 μM was chosen as the minimal concentration for reliable results.

3. Results

The *proenkephalin* gene is induced in primary striatal neurons after a 6-h treatment with the L-type Ca^{2+} channel agonist FPL 64176 (20 μM), as shown in Northern blots (Fig. 1A; the experiment was repeated four times). This induction is prevented by pretreatment for 30 min and co-treatment for 6 h with the L-type Ca^{2+} channel antagonist nifedipine (20 μM). Similarly, the transfected *pENKAT12* construct, which contains the *proenkephalin* promoter linked to chloramphenicol acetyltransferase [4], was induced by FPL 64176 (20 μM) after a 6-h treatment, and inhibited by pre- and co-treatment with nifedipine (20 μM; Fig. 1B). The Δ80 construct was not responsive to FPL 64176, whereas the −772 construct yielded similar results to *pENKAT12*, albeit with a lower fold-induction by FPL 64176 (4.5-fold, not shown). Because the Δ80 construct was not responsive to FPL 64176, the area of activation of the proenkephalin enhancer can be narrowed to between 84 and 193 bp upstream of the transcription initiation site, to the previously described CRE1 and CRE2 sites [4,6].

Interestingly, induction of the *proenkephalin* gene by the adenylate cyclase stimulating agent, forskolin (10 μM), was also inhibited by nifedipine (20 μM; Fig. 2A; the experiment was repeated four times). Comparable data were obtained with the transfected *pENKAT12* construct (Fig. 2B) and the −772 construct (not shown), whereas the Δ80 construct was unresponsive. Forskolin-mediated *proenkephalin* expression was furthermore dependent on Ca^{2+}/calmodulin kinase (CaMK), as the CaMK II/CaMK IV inhibitor, KN62 (30 μM), inhibited forskolin-mediated *pENKAT12* induction (Fig. 2C). Similar data were observed in Northern blots with the native *proenkephalin* gene (not shown). Forskolin-mediated *proenkephalin* expression was also dependent on PKA, demonstrated by the inhibition of *pENKAT12* induction by the PKA antagonist H89 (20 μM; Fig. 2D). Northern blots with native *proenkephalin* expression confirmed the findings (not shown). These data demonstrate that the induction of the *proenkephalin* gene depends on PKA as well as on CaMK.

Proenkephalin gene expression and *pENKAT12* induction by the L-type Ca^{2+} channel agonist FPL 64176 was also blocked by KN62 (30 μM; Fig. 3A,B). Surprisingly, FPL 64176-mediated *proenkephalin* expression (Fig. 3C) and *pENKAT12* induction (Fig. 3D) were inhibited by H89, demonstrating a dependence of L-type Ca^{2+} channel-mediated *proenkephalin* expression in striatal neurons on PKA.

4. Discussion

In molecular models of striatal function, cyclic AMP pathways play an important role. The high density of G-protein coupled receptors, many of which are coupled to adenylyl cyclase, attracts attention to the role and function of cyclic AMP-mediated gene expression in striatal neurons. The *proenkephalin* gene is a perfect candidate to study cyclic AMP-mediated gene expression in the striatum, as it codes for a neuromodulator with documented functions, is located in the striatum, and is sensitive to changes in cyclic AMP levels [4,5,14,15]. Among the G-protein coupled receptors in the striatum are dopamine and adenosine receptors, both of which regulate levels of *proenkephalin* mRNA and enkephalin protein [12,14,15,17,20]. The most obvious increase in demand for enkephalin is during times of increased neuronal

activity and, thus, increased enkephalin release, raising the question of how the cyclic AMP second messenger pathway synchronizes with neuronal activity. We show here that *proenkephalin* mRNA synthesis depends on L-type Ca^{2+} channel activity and on CaMK. Blockade of L-type Ca^{2+} channels, or inhibition of CaMK, disrupts PKA-stimulated *proenkephalin* mRNA synthesis. Thus, the cyclic AMP second messenger pathway can induce *proenkephalin* mRNA synthesis only in an active neuron. Because we do not add depolarizing agents with forskolin, our data suggest furthermore that the cyclic AMP pathway increases neuronal excitability to agents already present in the medium, such as KCl (5 mM) and glutamate (1–5 μM) [16]. A possible mechanism might be the phosphorylation of PKA sites on ion channels such as the L-type Ca^{2+} channel [2,9]. The notion that PKA contributes to neuronal excitability via modulation of ion channels is supported by our observation that inhibition of PKA prevents L-type Ca^{2+} channel-mediated induction of *proenkephalin*. The data suggest that basal phosphorylation of PKA sites is necessary for L-type Ca^{2+} channel-mediated *proenkephalin* expression, and increased phosphorylation might increase the likelihood that a neuron depolarizes. Taken together, we provide a model by which cyclic AMP-mediated gene induction is synchronized with neuronal depolarization. Increased PKA activity, as well as activation of L-type Ca^{2+} channels, is needed for the induction of *proenkephalin* gene expression. Indeed, a co-dependency of the L-type Ca^{2+} channel pathway with the PKA pathway provides a dual checkpoint for *proenkephalin* gene expression.

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Abbreviations

PKA	protein kinase A
CaMK	Ca^{2+}/calmodulin kinase

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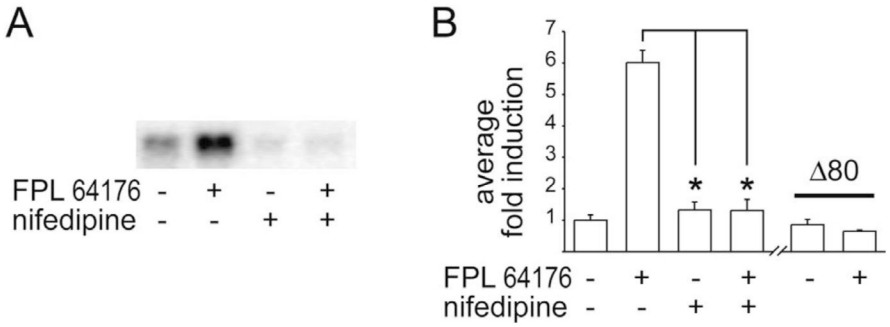

Fig. 1.

The L-type Ca^{2+} channel agonist FPL 64176 induces proenkephalin synthesis in primary striatal culture. (A) *Proenkephalin* gene levels are upregulated by the L-type Ca^{2+} channel agonist FPL 64176 (20 μM). This upregulation is blocked by the L-type Ca^{2+} channel antagonist nifedipine (20 μM). (B) The *proenkephalin* construct *pENKAT12*, transfected into primary striatal neurons, is induced by FPL 64176 (20 μM). The induction is blocked by nifedipine (20 μM). The construct with a shorter promoter, Δ80, is not induced by FPL 64176. Average fold induction of n=4–7±S.E.M. is shown. *P<0.001.

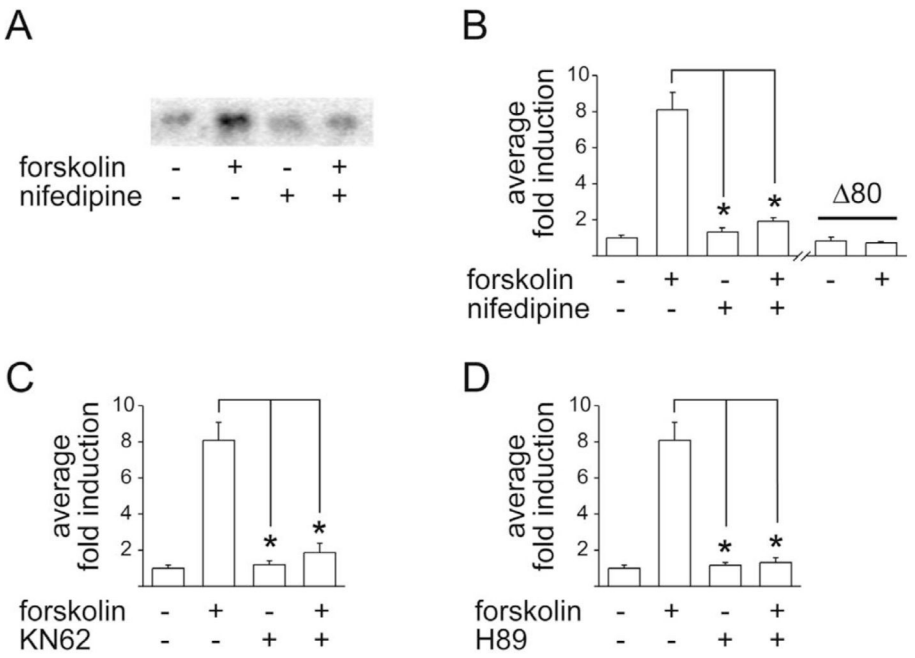

Fig. 2.

Proenkephalin induction by forskolin is inhibited by the L-type Ca^{2+} channel antagonist nifedipine, by the CaMK inhibitor KN62, and by the PKA inhibitor H89. (A) Forskolin (10 μM)-induced expression of the *proenkephalin* gene is blocked by nifedipine (20 μM). (B) The *proenkephalin* construct *pENKAT12*, transfected into primary striatal neurons, is induced by forskolin (10 μM). The induction is blocked by nifedipine (20 μM). The Δ80 construct is not induced by forskolin. (C) *pENKAT12*-induction by forskolin (10 μM) is blocked by KN62 (30 μM), and by H89 (20 μM), (D). Average fold induction of n=7–10±S.E.M. is shown. * P<0.001.

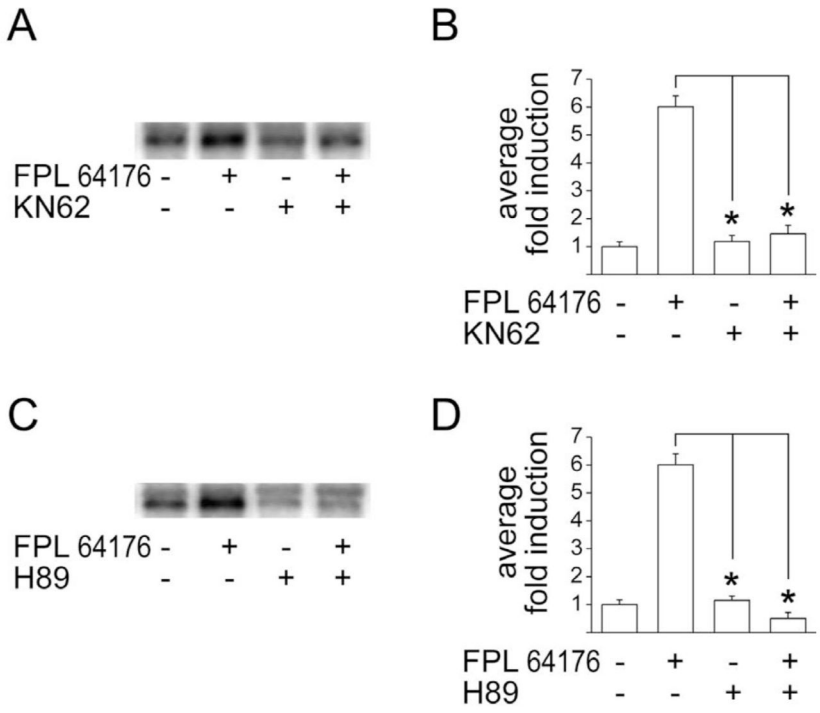

Fig. 3.

Proenkephalin induction by FPL 64176 is blocked by the CaMK inhibitor KN62 and by the PKA inhibitor H89. (A) FPL 64176 (20 μM)-mediated induction of the *proenkephalin* gene is blocked by KN62 (30 μM). (B) FPL 64176 (20 μM)-mediated induction of the *proenkephalin* construct *pENKAT12* is blocked by KN62 (30 μM). (C) FPL 64176 (20 μM)-mediated induction of the *proenkephalin* gene is blocked by H89 (20 μM). (D) *pENKAT12*-induction by FPL 64176 (20 μM) is blocked by H89 (20 μM). Average fold induction of n=4–7±S.E.M. is shown. * P<0.001.