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UNC80 functions as a scaffold for Src kinases in NALCN channel function

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Abstract

Ion channels can be regulated by a wide spectrum of neurotransmitters and hormones, largely through G-protein-coupled receptors (GPCRs). G-protein-independent activation of ion channel currents by GPCRs has also been recorded, although the molecular identity of the channels and the activation mechanisms remain largely unknown. UNC80 is a protein that is associated with the NALCN Na^+ leak cation channel, and is required for the activation of this channel by the neuropeptide substance P through GPCRs in a G-protein-independent fashion. Here, we show that UNC80 binds Src kinases and recruits Src into the channel complex. This finding is consistent with the known requirement for Src kinases in the activation of NALCN, and may lead to new insights into the molecular mechanisms underlying G-protein-independent activation of the channel.

Keywords

NALCN; UNC80; scaffold; Src kinases; cation channel

INTRODUCTION

Many hormones and neurotransmitters regulate ion channels through metabotropic G-protein-coupled receptors (GPCRs) [1]. This indirect control of ion channels provides a mechanism to fine-tune physiological properties such as neuronal excitability and heart rate. GPCRs are usually coupled to ion channels through the trimeric G proteins (Gα, Gβγ), although the way in which the signal is transmitted to the channels through G proteins can vary. For example, the βγG-protein subunits can directly gate K^+ channels [2]. Alternatively, Gα can activate downstream effectors such as phospholipase C, which subsequently affect a range of additional ion channels, including the KCNQ channels, several nonselective TRP channels, voltage-gated CaV channels, and the Ca^{2+}-release- activated Ca^{2+} channels [1, 3–9].

In addition to the extensively studied G-protein-dependent regulation of ion channels by GPCRs, patch clamp recording data have suggested that a second class of GPCR-activated cation channel currents may exist that are independent of G-protein activation. Specifically, inclusion of GDP-β-S in the pipette, which locks the G-proteins in an inactive state, does not inhibit the currents. Such atypical GPCR activation of cation currents has been recorded from cardiac myocytes, insulin-secreting β cells, and neurons from several brain areas (see [10] for review). In contrast to the better-established G-protein-dependent GPCR-activated ion channels, little is known about the molecular identities of the G-protein-independent cation channels, their mechanism of activation, or their physiological significance. We recently

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reported that NALCN, a Na^+-leak cation channel 11, can be activated by the neuropeptide substance P (SP) through GPCRs in a G-protein-independent manner, both in neurons and in HEK293T cells expressing the receptor-channel complex [12]. The mechanism by which this occurs is not clear, but the observation that SFK inhibitors eliminate channel activation suggests a requirement for the Src family kinases (SFK). Activation of NALCN also requires UNC80, a mammalian homolog of the *C. elegans* Unc-80 protein. UNC80 is a novel, large protein (3326 amino acids in mice) that lacks an obvious functional domain [12–15]. How UNC80 participates in the G-protein-independent activation of NALCN channel by GPCRs is not yet known. In this report, we show that UNC80 acts as a scaffold for Src, and recruits Src into the NALCN channel complex.

RESULTS AND DISCUSSION

UNC80 is physically associated with NALCN, and is required for the channel's activation by SP in HEK293T cells transfected with NALCN, UNC80, and the SP GPCR receptor TACR1 [12]. In addition, both UNC80 and NALCN are tyrosine-phosphorylated. To test whether UNC80 directly interacts with Src kinases, we cotransfected cells with a combination of UNC80 and either wild-type Src, a constitutively active Src (Src529, with a Y529F mutation), or a dominant-negative Src (Src296, with double mutations of Y529F and K296R). Immunoprecipitation of Src or Src529 with an anti-Src antibody also precipitated UNC80 (Fig. 1, left panel). In a reciprocal experiment, immunoprecipitation of UNC80 with an anti-UNC80 antibody also precipitated Src and Src529 (Fig. 1, right panel). These data suggested that UNC80 can physically interact with Src and Src529. In contrast, the inactive Src mutant Src296, which reacted with the anti-Src antibody, did not appear to associate with UNC80.

The channel pore-forming protein NALCN is also tyrosine-phosphorylated [12]. When co-transfected with Src, NALCN (FLAG-tagged) could be brought down with an anti-Src antibody, suggesting that the two may physically interact (Fig. 2, lane 6). When UNC80, which does not increase the total amount of NALCN in HEK293T cells (not shown), was added to the complex, the amount of NALCN protein precipitated by anti-Src was much larger, possibly because UNC80 recruited more Src into the NALCN complex (compare lanes 2 and 6 with lanes 1 and 5, respectively.) Again, the dominant negative Src did not interact with the complex.

Since tyrosine phosphorylation is required for the channel activation by SP [12] and the dominant negative Src mutant that was unable to cause tyrosine phosphorylation also failed to interact with UNC80 and NALCN, we wondered whether tyrosine phosphorylation is required for the physical interaction between Src and the channel complex. Immunoprecipitation was carried out after cells were treated with PP1, an SFK inhibitor that inhibits tyrosine phosphorylation of UNC80 and NALCN, as well as channel activation [12]. PP1 treatment did not affect the physical association between Src and UNC80 or NALCN, suggesting that tyrosine phosphorylation is not required for the interaction (Fig. 3).

Taken together, our data suggest that UNC80 can interact with Src, and that the amount of Src associated with the NALCN channel complex is increased in the presence of UNC80. Src normally binds its substrates through its SH2/SH3 domains [16]. Intriguingly, Src296 does not interact with UNC80, suggesting that regions outside the SH2/SH3 domains may be involved in the UNC80-Src interaction. Further studies will examine how the recruitment of Src by UNC80 into the channel complex is involved in signal transduction in the G-protein-independent activation by GPCRs of the channel, and what role this might normally play in neuronal function.

Acknowledgments

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Abbreviations and Acronyms

GPCR	G-protein coupled receptor
SFKs	Src family of tyrosine kinases

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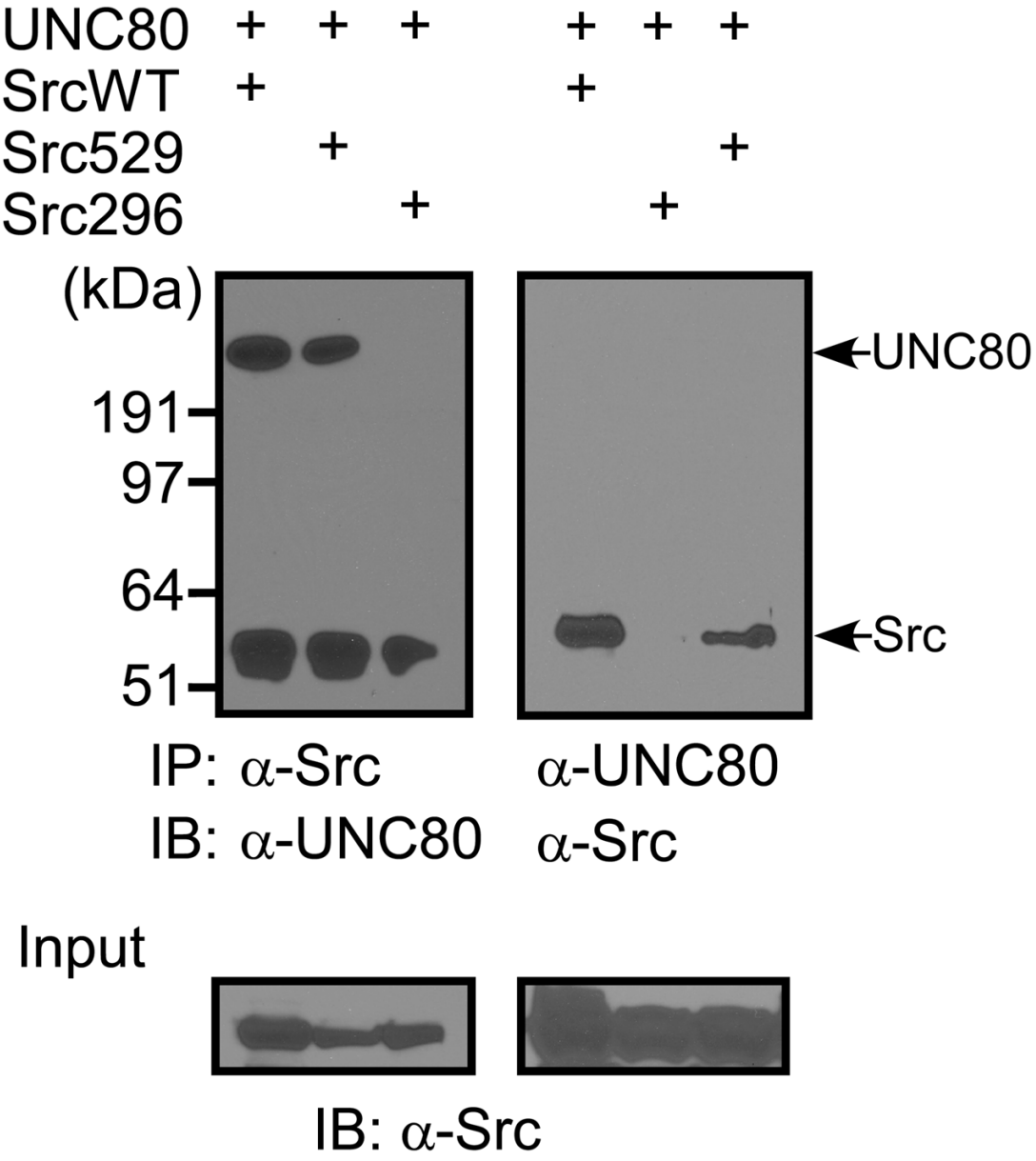

Figure 1. UNC80 interacts with Src

Upper left panel: Total lysates from HEK293T cells transfected as indicated were immunoprecipitated with anti-Src (monoclonal, # SC-8056 from Santa Cruz) and blotted with anti-UNC80 [12], showing that wild-type (SrcWT) and activated Src (Src529), but not dominant negative Src (Src296), coimmunoprecipitated with UNC80. Upper right panel: In the reverse experiment, anti-UNC80 precipitated SrcWT and Src529, but not Src296. Lower panel: inputs were blotted with anti-Src showing that the antibody recognized both the wild-type and the mutant Src proteins.

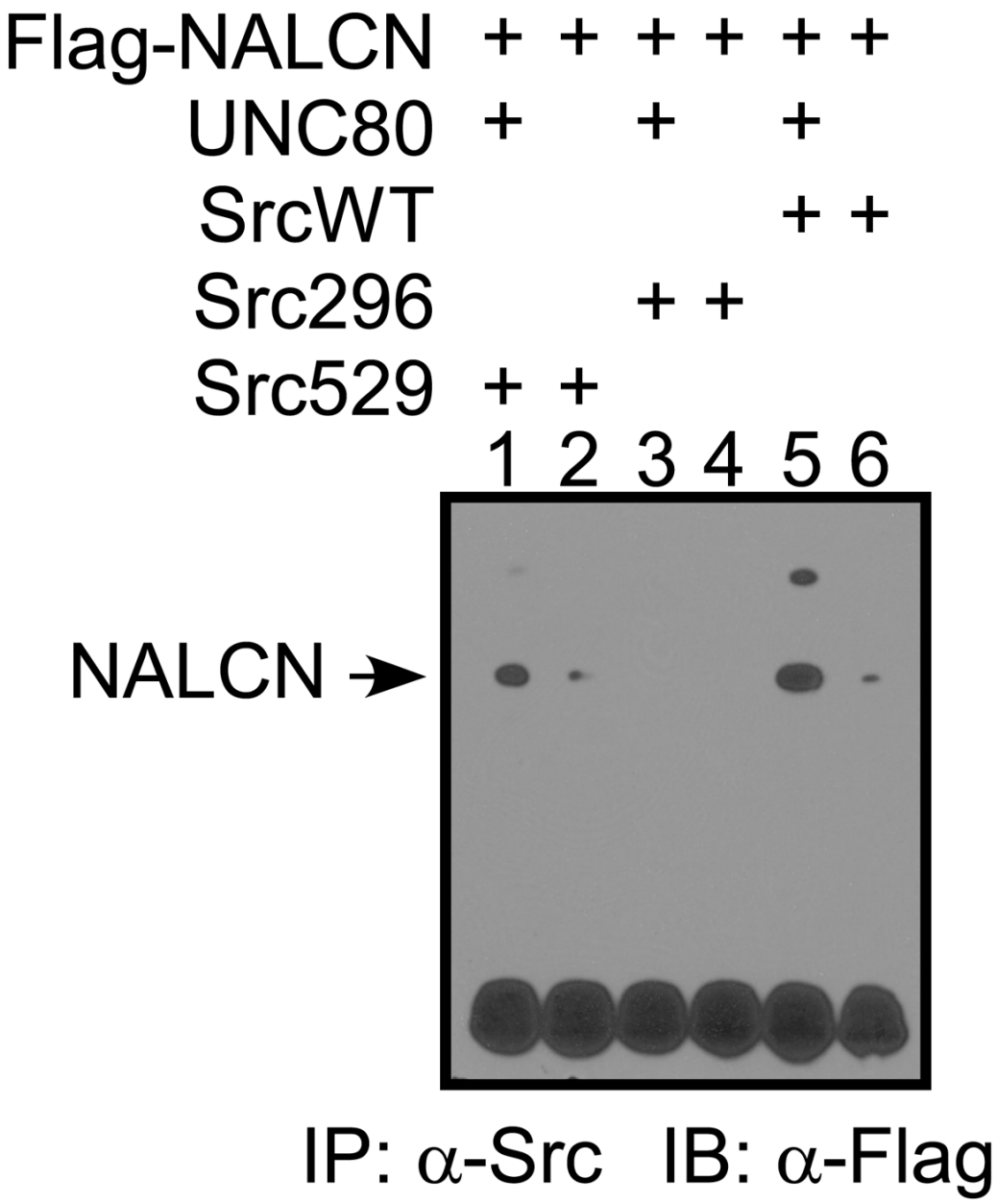

Figure 2. UNC80 recruits Src into the NALCN channel complex

Lysates from HEK293T cells co-transfected with Flag-NALCN and Src, with or without UNC80, were immunoprecipitated using anti-Src antibody and then detected with anti-Flag. Src529 and SrcWT, but not Src296, caused NALCN to be precipitated by anti-Src antibody (lanes 2, 6, and 4, respectively). When cells were cotransfected with UNC80, a much larger amount of NALCN coimmunoprecipitated with Src529 and SrcWT (lanes 1 and 5).

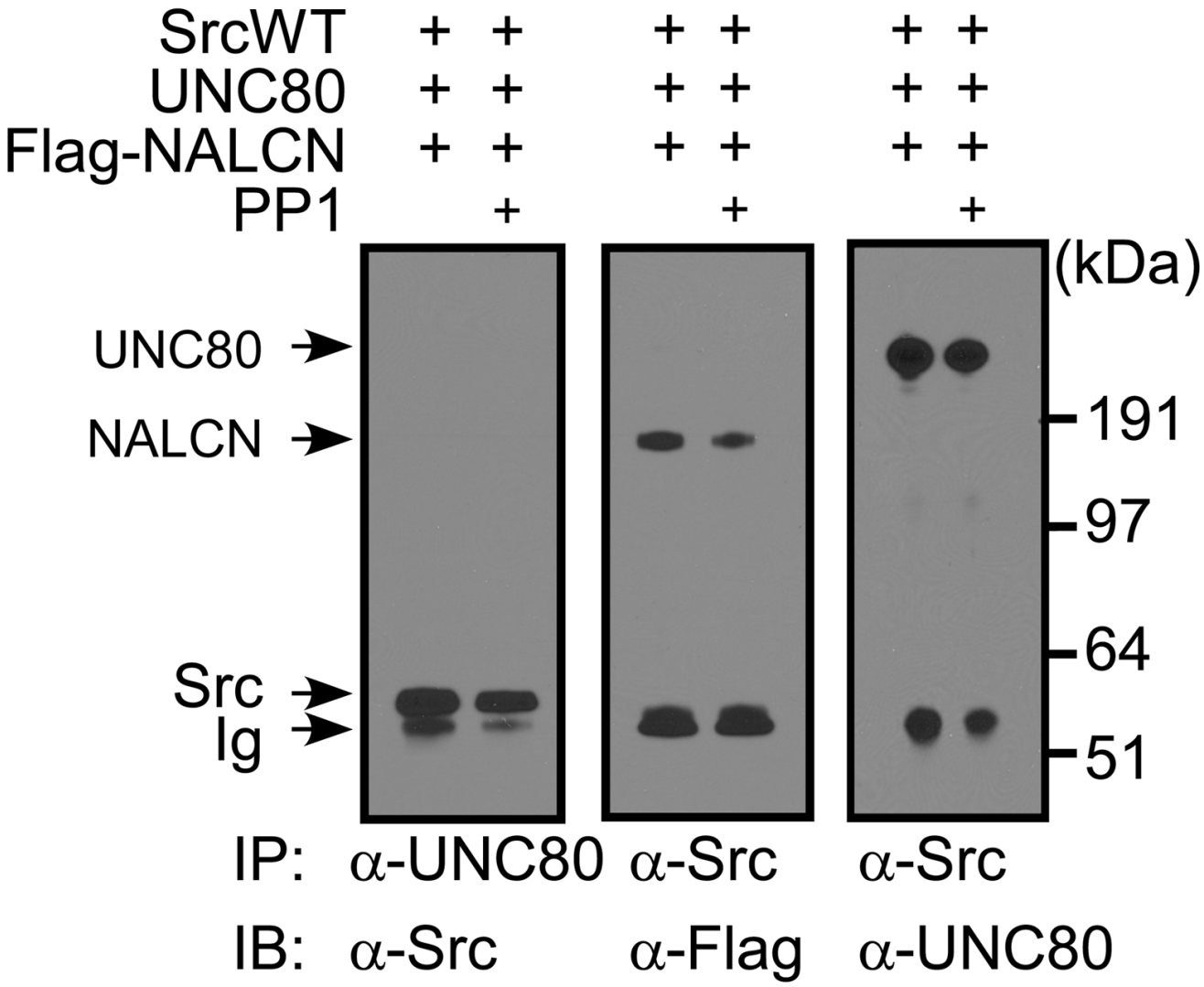

Figure 3. The interaction is independent of tyrosine phosphorylation

HEK293T cells overexpressing Flag-NALCN, UNC80, and Src proteins were either untreated or treated with the SFK inhibitor PP1 (10 μM) for 2 h prior to immunoprecipitation with anti-UNC80 or anti-Src. Precipitates were probed with the indicated antibodies. UNC80, NALCN, Src, and Ig bands are indicated with arrows.