Purinergic stimulation of K\(^+\)-dependent Na\(^+\)/Ca\(^{2+}\) exchanger isoform 4 requires dual activation by PKC and CaMKII

Xu Yang* and Jonathan Lytton*1

*Libin Cardiovascular Institute of Alberta and Hotchkiss Brain Institute, Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta T2N 4N1, Canada

**Correspondence should be addressed (email: jlytton@ucalgary.ca).

Abbreviations used: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; [Ca\(^{2+}\)]\(_i\), concentration of free ionized calcium; CaMKII, Ca\(^{2+}\)-/calmodulin-dependent protein kinase II; ER, endoplasmic reticulum; HEK-293, human embryonic kidney cell; HEK-293T, HEK-293 cells expressing the large T-antigen of SV40 (simian virus 40); NCKX, K\(^+\)/Ca\(^{2+}\)-exchanger; NCX, Na\(^+\)/Ca\(^{2+}\)-exchanger isoform 4 (NCX4); P2Y, purinergic receptor; PKC, protein kinase C; PLC, phospholipase; Tg, thapsigargin.

INTRODUCTION

A transient increase in intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) is required for a wide range of signalling events, especially in excitable cells such as neurons. Since Ca\(^{2+}\) cannot be metabolized, an increase in [Ca\(^{2+}\)]\(_i\) must be balanced by subsequent extrusion processes. In excitable cells where Ca\(^{2+}\) fluxes are high, this extrusion is predominantly mediated by Na\(^+\)/Ca\(^{2+}\)-exchangers, which have a high rate of activity, and best exemplified by the cardiac exchanger, NCX1. Recent studies confirm that NCKX4 plays a critical role maintaining Ca\(^{2+}\) homeostasis underlying adaptation and termination in olfactory neurons [12], and in the normal production of tooth enamel [13]. Extensive studies on cardiac NCX1 have established a complex array of regulatory processes that involve the central cytosolic loop of that protein and which depend on the binding of Ca\(^{2+}\), five transmembrane spans) joined by a central cytoplasmic loop of varying length. Sequence similarity among members is highest within the transmembrane spans and lowest in the cytoplasmic loops [6–8]. NCKX proteins are also more distantly related to the NCX (Na\(^+\)/Ca\(^{2+}\)-exchanger) (SLC8) family of Na\(^+\)/Ca\(^{2+}\)-exchangers, best exemplified by the cardiac exchanger, NCX1 [9]. The structure of a bacterial Na\(^+\)/Ca\(^{2+}\)-exchanger homologue was recently published, confirming previous topological models for NCKX proteins, and the location of the ion binding and transport sites in the membrane segments [10]. Within brain neurons, NCKX2, NCKX3 and NCKX4 are all expressed with different abundance in different regions [11]. Recent experiments confirm that NCKX4 plays a critical role maintaining Ca\(^{2+}\) homeostasis underlying adaptation and termination in olfactory neurons [12], and in the normal production of tooth enamel [13].

Synopsis

K\(^+\)-dependent Na\(^+\)/Ca\(^{2+}\)-exchanger isoform 4 (NCX4) is one of the most broadly expressed members of the NCKX (K\(^+\)-dependent Na\(^+\)/Ca\(^{2+}\)-exchanger) family. Recent data indicate that NCX4 plays a critical role in controlling normal Ca\(^{2+}\) signal dynamics in olfactory and other neurons. Synaptic Ca\(^{2+}\) dynamics are modulated by purinergic regulation, mediated by ATP released from synaptic vesicles or from neighbouring glial cells. Previous studies have focused on modulation of Ca\(^{2+}\) entry pathways that initiate signalling. Here we have investigated purinergic regulation of NCX4, a powerful extrusion pathway that assists in terminating Ca\(^{2+}\) signals. NCX4 activity was stimulated by ATP through activation of the P2Y receptor signalling pathway. Stimulation required dual activation of PKC (protein kinase C) and CaMKII (Ca\(^{2+}\)-/calmodulin-dependent protein kinase II). Mutating T312, a putative PKC phosphorylation site on NCX4, partially prevented purinergic stimulation. These data illustrate how purinergic regulation can shape the dynamics of Ca\(^{2+}\) signalling by activating a signal damping and termination pathway.

Key words: Ca\(^{2+}\)-/calmodulin-dependent kinase II (CaMKII), K\(^+\)-dependent Na\(^+\)/Ca\(^{2+}\)-exchanger isoform 4 (NCX4), P2Y-purinergic receptor, protein kinase C (PKC).

Cite this article as: Yang, X. and Lytton, J. (2013) Purinergic stimulation of K\(^+\)-dependent Na\(^+\)/Ca\(^{2+}\) exchanger isoform 4 requires dual activation by PKC and CaMKII. Biosci. Rep. 33(6), art:e00087.doi:10.1042/BSR20130099
Na⁺, H⁺, anionic phospholipids and other factors [5,9]. Regulation among members of the NCKX family has not been studied in as much detail, but evidence has been presented indicating that Na⁺ occupancy of the transport sites can lead to time-dependent inhibition of function for NCKX2 [14], whereas studies using phorbol esters have established that PKC (protein kinase C) can stimulate NCKX2, but not NCKX3 or NCKX4 [15].

ATP, a purinergic agonist, has long been described as an important neurotransmitter and neuromodulator that regulates synaptic [Ca2⁺], dynamics [16,17]. ATP, released either from glial cells [18] or from synaptic vesicles [19], activates purinergic P2X and P2Y receptors. P2X receptors are ligand-gated ion channels, while P2Y receptors are G-protein-coupled receptors that signal through either cAMP/protein kinase A or inositol trisphosphate/DAG (diacylglycerol)/Ca2⁺ depending on the molecular sub-type of the receptor and the G protein it couples to: Gαi/Go or Gq/11 [20]. During neuronal stimulation, ATP and other excitatory neurotransmitters are co-released to exert co-modulation at the post-synaptic terminal, resulting in activation of a rapid ‘on’ switch that raises [Ca2⁺], and a slower ‘off’ process that is associated with Ca2⁺ extrusion to ensure proper [Ca2⁺], homeostasis [19]. While many studies have focused on the influence that purinergic signalling has on Ca2⁺ entry processes that initiate signals [20–24], little is known about regulation of extrusion process that serve to dampen and/or terminate Ca2⁺ signals. Na⁺/Ca2⁺-exchangers, particularly those of the NCKX class, play an important role in the Ca2⁺ extrusion process [4,25,26]. While it seems probable that the efflux mediated by exchangers like NCKX4 would be regulated, such that activity is low during the early signal initiation phase and higher during the later termination phase, this has not been investigated previously. We hypothesized that purinergic signalling might be the mechanism providing such physiological regulation.

In the present study, we investigated the link between purinergic signalling and NCKX4 activity in transfected human embryonic kidney HEK-293T [HEK-293 cells expressing the large T-antigen of SV40 (simian virus 40)] cells, and found that NCKX4 activity can be stimulated by ATP through a P2Y-dependent signalling pathway. We demonstrated, using pharmacological interventions, that stimulation of NCKX4 requires the simultaneous activation of CaMKII (Ca2⁺/calmodulin-dependent protein kinase II) and PKC. Mutation of one candidate PKC site, T312, significantly reduced the extent of this stimulation.

### Cell culture and transfection
All cell culture reagents were obtained from Invitrogen (invitrogen.com). HEK-293 cells [HEK-293T] were cultured in DMEM (Dulbecco’s modified Eagle’s medium) containing 10% (v/v) FBS, 1% (v/v) L-glutamine, 1% non-essential amino acids, and maintained in 5% (v/v) CO2 at 37°C. Transfection of Qiagen purified expression plasmid into HEK-293 cells was performed according to a previously published standard Ca2⁺-phosphate precipitation protocol [27]. Cells transfected with the parent expression vector, pcDNA3.1+ (Invitrogen), were used as negative controls.

### cDNA constructs and mutagenesis
Mouse NCKX4 cDNA in the vector pcDNA3.1 (+) was used for all experiments described here. This construct was based on GenBank accession AY156046, and included a 72 nucleotide 5’-end extension, as present in GenBank accession AK44368, encoding an additional 17 amino acids at the N-terminus of the NCKX4 protein. In addition, the current clone included a silent C for G replacement at amino acid residue 44, and lacked the exon corresponding to the alternatively spliced region (amino acids 275–293). Site-directed mutagenesis was performed to create T256A, T312A, S325A, T256A/T312A and T256A/T312A/S325A mutants using the QuikChange II Site-Directed Mutagenesis Kit from Agilent Technologies (agilent.com), according to the manufacturer’s protocol. Each NCKX4 mutant was sequenced to ensure only the intended change was present in the construct.

### Photometry and data analysis
Transfection and analysis of NCKX transport function was performed essentially as reported previously [27]. In brief, 2 days following transfection, HEK-293 cells grown on coverslips were loaded with 5 μM fura-2 AM (Invitrogen) and mounted in a perfusion chamber on the stage of an Olympus (olympuscanada.com) IX50 microscope, and observed using a 20X UAPO/340 0.75NA objective. A D-104 photometer (Photon Technology International; pti-nj.com) was used to measure fluorescence emission at 510 nm after fura-2 excitation at 340 and 380 nm, collected from a field of ~50–100 cells, typically transfected with a frequency of 20–30%. The resulting 340/380 fura-2 ratio was calculated in FelixGX software (Photon Technology International). Unlike NCKX2, NCKX4 possesses a relatively high apparent affinity for K⁺ [27]. When NCKX4 was measured under a standard KCl concentration of 5 mM, activity levels were too rapid to be resolved as discrete rates in our system. Consequently, we developed the following protocol where [KCl] was rate-limiting (0.25 mM), thereby allowing precise resolution of NCKX4 activity. Perfusion solutions containing 10 mM Hepes-tetramethylammonium, pH 7.4, 10 mM glucose, 0.1 mM CaCl₂ with either 145 mM NaCl (Na0K) or 144.75 mM LiCl and 0.25 mM KCl (Li0.25K) were used alternatively for 3 or 2 min intervals, respectively, at a rate of ~3 ml/min. The rates of Ca2⁺ transport during the reverse mode of NCKX4

### MATERIALS AND METHODS

#### Materials
All chemicals and reagents were purchased from Sigma-Aldrich (sigmaaldrich.com) or EMD (emdanada.com), and were analytical grade or higher, except where otherwise indicated. All molecular techniques were performed according to previously established protocols or manufacturer’s instructions, unless otherwise specified.
NCKX4 stimulation requires dual activation of PKC and CaMKII

Figure 1  A quantitative assay for NCKX4 activity

HEK-293 cells transfected with cDNA encoding mouse NCKX4, or vector alone, were loaded with fura-2, mounted in a perfusion chamber, and examined by microscopic photometry, as described in ‘Experimental Procedures’. (A) The fura-2 340/380 excitation ratio is plotted with time for either vector only-transfected cells (lower trace) or NCKX4-transfected cells (upper trace), both subjected to repeated perfusion switches between solution containing 145 mM NaCl (Na0K) or 144.75 mM LiCl and 0.25 mM KCl (Li0.25K). NCKX4 activity results in Ca2+ influx during the Li0.25K perfusion pulse, causing an increase in the fura-2 ratio. (B) The initial portion of each rising fura-2 ratio (pulses 2–7 from panel A) is shown expanded, with the steepest segment chosen for linear regression analysis boxed. (C) Individual data points and the linear regression fit for data from the boxed regions of panel (B), with the resulting slope values indicated. (D) The rates of fura-2 ratio rise (average ± S.E.M.), internally normalized within each experiment to the rate of pulse 2, are shown averaged from four independent experiments.

RESULTS

Development of a functional assay for recombinant NCKX4

HEK-293 cells grown on coverslips were transfected with recombinant constructs expressing mouse NCKX4 or vector alone as a negative control. Transfected cells loaded with fura-2 AM were subsequently mounted in a perfusion chamber on the microscope stage. When the perfusion solution containing 145 mM Na+, but no K+ (Na0K) was switched to one containing 144.75 mM Li+, 0.25 mM K+, but no Na+ (Li0.25K), a rapid increase in fura-2 ratio was observed in NCKX4-transfected HEK-293 cells but not in negative control (Figure 1A). The increase in fura-2 ratio also required the presence of K+ ions in the perfusion solution. Hence, this increase in fura-2 ratio represented Ca2+ influx mediated by reverse-mode NCKX4 activity. Data collected over repeated alternating perfusion switches between Na0K and Li0.25K for a total of six or seven pulses were then used to generate fura-2 versus time graphs in which the maximal initial rate of each individual pulse was determined based on linear regression analysis over a time frame of 10 s (Figures 1B and 1C). At noted above, the increase in fura-2 ratio was seen only when cells were transfected with constructs expressing NCKX4 (see Figure 1A). Furthermore, the initial rate of perfusion-induced fura-2 ratio increase was significantly greater than the subsequent rate of perfusion-induced decline at comparable fura-2 ratio values (Figure 1A). Thus, it can be concluded that these initial rates of fura-2 ratio increase corresponded to the rate of NCKX4-mediated Ca2+ influx at the time of the perfusion pulse. In all experiments, the rate for the first pulse was inconsistent with the subsequent pulses (although we have no explanation for this phenomenon, it is routinely observed in our laboratory [27]), and was therefore excluded from the analyses, and instead pulse two

© 2013 The Author(s) This is an Open Access article distributed under the terms of the Creative Commons Attribution Licence (CC-BY) (http://creativecommons.org/licenses/by/3.0/) which permits unrestricted use, distribution and reproduction in any medium, provided the original work is properly cited.
ATP treatment stimulates NCKX4 activity

HEK-293 cells transfected with cDNA encoding mouse NCKX4 were analysed as described in Figure 1. (A) A time-control trace illustrating the repeated activation protocol to measure NCKX4 activity. (B) Following pulse four, cells were perfused with solutions containing 0.2 mM ATP as illustrated. (C) HEK-293 cells transfected with vector only, subjected to the same alternating perfusion switches and ATP treatment seen in panel (B). (D) Normalized NCKX4 activity (rate of fura-2 rise) is averaged (±S.E.M.) for one independent trial from each of four different experiments (control; black diamonds) or two independent trials from each of four different experiments (ATP added at the arrow; red squares). Two-way ANOVA analysis revealed statistically significant differences in normalized rates between control and ATP-treated cells (P < 0.001; 0.001; 0.05 for pulses 5–7, respectively). The magnitude of the increase in NCKX4 activity induced by ATP was relatively consistent among different samples from one experiment, but ranged from 1.8- to 4-fold between different experiments over the duration of this study.

was used for internal rate normalization. Measured in this way, NCKX4 activity under control conditions was relatively stable with time and with respect to repeated perfusion pulses (Figure 1D).

Purinergic agonist treatment results in stimulation of NCKX4 activity

To ascertain if purinergic agonists such as ATP exert an influence on NCKX4 activity, four perfusion pulses were used to establish the baseline NCKX4 activity. At that point, the alternating perfusion solutions were switched to ones containing 0.2 mM ATP. Time-based control experiments in the absence of ATP were also conducted (Figure 2A). Shortly after introducing ATP into the system, a large Ca\(^{2+}\) transient was observed, which quickly returned to base level (Figure 2B). The first NCKX4-activating pulse following ATP addition displayed a dramatic increase in the magnitude of the fura-2 ratio compared with the baseline pulses, a change that gradually declined over subsequent pulses. The rate analysis of this set of experiments showed that on average, there was a 1.8-fold increase in NCKX4 activity following ATP stimulation (Figure 2D). ATP treatment of negative control cells (transfected with vector alone) elicited a large Ca\(^{2+}\) transient, but no Ca\(^{2+}\) changes in response to perfusion switches (Figure 2C). Hence, during the perfusion conditions employed in this experiment to measure NCKX4 activity in reverse, Ca\(^{2+}\) entry, mode, ATP treatment did not activate plasma membrane Ca\(^{2+}\) flux pathways other than NCKX4. Collectively, these data demonstrate that treatment with ATP stimulates NCKX4 activity. The magnitude of the stimulation induced by ATP was quite consistent from coverslip to coverslip within any given experiment, but varied significantly from day to day. Consequently, positive control experiments with ATP stimulation were performed in parallel with other treatments in all cases described below. Over the course of all the experiments described here, ATP stimulation resulted in increases in NCKX4 activity that ranged from 1.8- to 4-fold. It is also important to note that the absolute rates of fura-2 increase, while relatively consistent, varied significantly from coverslip to coverslip and from experiment to experiment. This variation is accommodated by normalizing the data to the baseline rate on each individual coverslip for all experiments.

The assay employed to measure NCKX4 used K\(^{+}\) at a rate-limiting concentration of 0.25 mM. Therefore it was unclear if ATP stimulation increased the \(V_{\text{max}}\) of NCKX4 or increased the
apparent affinity for K⁺. This issue was addressed by altering our assay protocol, so that Ca²⁺ was held at the rate-limiting concentration of 0.3 μM with an EGTA buffering system, while KCl was included at 5 mM. Under these conditions, ATP still resulted in a similar stimulation of NCKX4 activity (Figure 3). These data, in which ATP induced similar stimulation of NCKX4 activity when either K⁺ or Ca²⁺ was rate-limiting, suggest an effect on the Vₘₐₓ of transport activity.

**Activation of the P2Y receptor signalling pathway is necessary for NCKX4 stimulation**

To examine which purinergic receptor pathway (P2X or P2Y) was activated by ATP to stimulate NCKX4, we first employed selective agonists. ATP is thought to activate P2Y (seven transmembrane-span G-protein-coupled) receptors at sub-micromolar concentrations, whereas much higher ATP levels are needed to activate P2X (ligand-gated ion channel) receptors [20]. Thus, NCKX4-transfected cells were treated with a range of ATP concentrations (Figure 4A). While the maximum stimulation was observed already at 2 μM ATP, 0.2 μM did not induce stimulation of NCKX4 activity. However, the Ca²⁺ transient typically observed upon ATP treatment of cells was much lower with 0.2 μM treatment than the response seen with higher concentrations of ATP, suggesting that under the conditions employed here, low concentrations of ATP do not efficiently activate P2Y receptors. Therefore this treatment regime was not able to distinguish P2X from P2Y receptor function. Consequently, NCKX4 stimulation was tested with 5 μM UTP, a concentration thought to activate P2Y but not P2X receptors [28,29]. This treatment induced a significant stimulation, suggesting a requirement for P2Y receptor activity in the NCKX4 response (Figure 4B). Finally, the treatment with α,β-methylene ATP, considered to be a P2X-specific agonist [30], was tested. Figure 4(C) shows that at concentrations below 100 μM, α,β-methylene ATP did not stimulate NCKX4. At 100 μM, however, α,β-methylene ATP partially stimulated NCKX4 but also caused a Ca²⁺ transient that resembled the effect of ATP. This suggested that at such a high concentration, α,β-methylene ATP lost specificity for P2X receptors. On balance, while not conclusive, these data support a role for P2Y, but not for P2X, receptors in the stimulation of NCKX4 activity.

Since the results with selective purinergic agonists were ambiguous, we decided to interrogate the downstream signalling elements in the P2Y pathway using pharmacological interventions. P2Y receptors typically signal through the PLCγ–IP₃–PKC pathway. If NCKX4 stimulation depends on activation of this pathway, interfering with any of the signalling components should prevent or inhibit exchanger stimulation. Therefore fura-2-loaded NCKX4-transfected cells were pre-incubated for 15 min in bathing solution that contained either a PLC inhibitor (U73122) or broad range PKC inhibitors (chelerythrine chloride or calphostin C) and subsequently tested for ATP-induced stimulation. Figure 5 shows that inhibition of either PLC or PKC abolished the stimulation following ATP treatment, indicating that ATP requires (likely via P2Y receptor signals) the activation of PLCγ and subsequently PKC to stimulate NCKX4.

**PKC activation alone is not sufficient for NCKX4 stimulation**

Since inhibitors of PKC prevented the stimulation of NCKX4, we next examined whether direct activation of PKC was sufficient for NCKX4 stimulation. However, neither phorbol esters PMA nor PDBu (phorbol 12,13-dibutyrate) at 100 nM were able to stimulate NCKX4, in sharp contrast to the robust stimulation observed for NCKX2, used as a positive control (Figure 6A). This stimulatory effect of phorbol ester alone on NCKX2 but not NCKX4 activity is consistent with our earlier studies [15]. We considered two possible explanations for the lack of phorbol ester-induced stimulation of NCKX4. First, it is possible that a ‘classical’ PKC isoform is necessary for stimulation of NCKX4, which under the
Figure 4  Examining purinergic specificity for stimulation of NCKX4

HEK-293 cells transfected with cDNA encoding mouse NCKX4 were analysed as described in Figure 2. (A) Cells were treated with different concentrations of ATP as illustrated in the different panels. Control, \( n = 4; \) 0.2\( \mu M \) ATP, \( n = 7; \) 2\( \mu M \) ATP, \( n = 6. \) Addition of ATP is indicated by the arrow. Two-way ANOVA analysis revealed statistically significant differences in normalized rates between control and 2\( \mu M \) ATP (\( P < 0.05 \) for pulses 4 and 5) or 20\( \mu M \) ATP-treated cells (\( P < 0.001, 0.01; 0.05 \) for pulses 4–6, respectively), but not 0.2\( \mu M \) ATP-treated cells. (B) Cells were treated with (ii) or without (i) 5\( \mu M \) UTP. Control, \( n = 2; \) UTP, \( n = 6. \) Addition of 5\( \mu M \) UTP is indicated by the arrow. Two-way ANOVA analysis revealed statistically significant differences in normalized rates between control and UTP-treated cells (\( P < 0.05 \) for pulses 4–6). (C) Cells were treated with different concentrations of the P2X-selective agonist, \( \alpha, \beta \)-methylene ATP (\( \alpha, \beta \)-MeATP) as illustrated. Control, \( n = 3; \) 0.2\( mM \) ATP, \( n = 2; \) 1\( \mu M \) \( \alpha, \beta \)-MeATP, \( n = 4; \) 10\( \mu M \) \( \alpha, \beta \)-MeATP, \( n = 6; \) 100\( \mu M \) \( \alpha, \beta \)-MeATP, \( n = 5. \) Addition of ligands is indicated by the arrow. Two-way ANOVA analysis revealed statistically significant differences in normalized rates between control and 0.2\( mM \) ATP (\( P < 0.001 \) for pulses 4–6) or 100\( \mu M \) \( \alpha, \beta \)-MeATP-treated cells (\( P < 0.01, 0.001; 0.001 \) for pulses 4–6, respectively) but not 1\( \mu M \) or 10\( \mu M \) \( \alpha, \beta \)-MeATP-treated cells.

conditions of our experiments cannot be activated by phorbol ester alone without a concomitant increase in \([Ca^{2+}]_i \) (NCKX2 stimulation induced with PMA has been shown to depend on PKC-\( \varepsilon \), a ‘novel’ non-Ca\(^{2+} \)-dependent isoform [15]). However, previously published work suggested that PMA alone is sufficient to activate even ‘classical’ PKC isoforms [31,32]. Thus, an alternative possibility is that stimulation of NCKX4 requires a second event in addition to PKC activation, possibly involving a Ca\(^{2+} \) signal. To test these ideas, NCKX4-transfected cells were treated with PMA and Tg (thapsigargin), an inhibitor of the SERCA (sarcoplasmic/endoplasmic reticulum Ca\(^{2+} \)-ATPase) [33]. As shown in Figure 6(B), a robust stimulation of NCKX4 activity, greater even in magnitude than the stimulation seen with ATP treatment, was observed when Tg and PMA were applied together, but not with PMA alone. Interestingly, Tg alone was able to induce a significant change in the rate of NCKX4 transport, albeit not as large as that produced by Tg and PMA co-treatment. This result suggests a possible Ca\(^{2+} \)-dependent second signalling event, in addition to PKC activation, required for NCKX4 stimulation.

CaMKII activation is required for NCKX4 stimulation

The fact that Tg, when used alone, was also able to partially stimulate NCKX4 suggested the involvement in regulation of a Ca\(^{2+} \)-dependent pathway distinct from PKC activation. Ca\(^{2+} \)
NCKX4 stimulation requires dual activation of PKC and CaMKII

Figure 5  Inhibition of PLC or PKC prevents NCKX4 stimulation
HEK-293 cells transfected with cDNA encoding mouse NCKX4 were stimulated with either 2 μM (panel A) or 0.2 mM ATP (panel B), added at the arrow, and analysed as described in Figure 2. Prior to initiation of the experiments, cells were pre-treated for 15 min with either 5 μM PLC inhibitor U73122, or the PKC inhibitors, chelerythrine chloride (5 μM) or calphostin C (100 nM), as indicated. (A) Control, n = 4; ATP, n = 5; ATP + U73122, n = 4; ATP + chelerythrine chloride, n = 4. Two-way ANOVA analysis revealed statistically significant differences in normalized rates between ATP-stimulated cells and cells pretreated with U73122 or chelerythrine chloride prior to ATP stimulation (U73122: P < 0.001; 0.01 for pulses 5 and 6, respectively; chelerythrine chloride: P < 0.001; 0.05 for pulses 5 and 6, respectively). (B) Control, n = 3; ATP, n = 4; ATP + calphostin C, n = 8. Two-way ANOVA analysis revealed statistically significant differences in normalized rates between ATP-stimulated cells and cells pretreated with calphostin C prior to ATP stimulation (P < 0.001; 0.05; 0.01 for pulses 4–6, respectively).

Figure 6  The effect of phorbol esters on NCKX4 activity
Transfected HEK-293 cells were treated with various reagents, added at the arrow and maintained in all solutions thereafter, and analysed as described in Figure 2. (A) Cells were transfected with cDNA encoding NCKX4 and treated either with vehicle alone (control; n = 3), 100 nM PMA (n = 5), 100 nM PDBu (n = 4), or NCKX2-transfected cells were treated with 100 nM PMA (n = 2). Two-way ANOVA analysis revealed statistically significant differences in the normalized rates between control and PMA-treated NCKX2-transfected cells (P < 0.001; 0.001; 0.01 for pulses 4–6, respectively), but not in PMA- or PDBu-treated NCKX4-transfected cells. (B) Cells were transfected with cDNA encoding NCKX4 and treated either with vehicle alone (control; n = 5), 0.2 mM ATP (n = 6), 0.5 μM Tg (n = 14), 100 nM PMA (n = 3) or Tg + PMA (n = 12). Two-way ANOVA analysis revealed statistically significant differences in normalized rates between control and ATP- (P < 0.001 for pulses 4–6), Tg- (P < 0.001; 0.05 for pulses 4 and 6, respectively) or Tg + PMA-treated cells (P < 0.001 for pulses 4–6), but not PMA-treated cells. Statistically significant differences were also found between ATP- and Tg + PMA-treated cells (P < 0.001; 0.05 for pulses 4 and 5, respectively), and between Tg- and Tg + PMA-treated (P < 0.001; 0.01 for pulses 4 and 5, respectively).

release from the ER, mediated by either IP3 or Tg, can trigger the activation of CaMKII. Hence, we carried out additional pharmacological intervention-based functional assays to evaluate this possibility. As shown in Figure 7(A), the CaMKII inhibitor, KN-93, but not its inactive congener, KN-92, effectively prevented Tg + PMA-induced NCKX4 stimulation. KN-93 was also effective in inhibiting ATP-induced stimulation of NCKX4 (Figure 7B). Furthermore, the ‘classical’ PKC inhibitor Go6983, was able to block stimulation of NCKX4 induced by Tg + PMA treatment, as well as stimulation by ATP (Figure 7). These data indicate that purinergic stimulation of NCKX4 requires activation of both PKC and CaMKII pathways, while blocking either PKC or CaMKII is sufficient to prevent purinergic stimulation.

Mutagenesis of a putative PKC phosphorylation site in the central cytoplasmic loop of NCKX4 partially prevented purinergic agonist-mediated stimulation
Based on the idea that regulation of NCKX4 is associated with kinase modulation of specific regulatory sites, we screened NCKX4 for putative PKC phosphorylation sites
NCKX4, we created both a double (T256A/T312A) and a triple sites could further prevent purinergic-induced enhancement of single mutants (T256A and S325A) were without effect. To the stimulatory effect of ATP by 50%, whereas the other two individual NCKX4 mutants. Figure 8 shows that T312A reduced S325, within a putative multi-phosphorylation motif for PKC, candidates (T256 and T312) in the central cytoplasmic loop of putative phosphorylation sites solely responsible for purinergic stimulation of NCKX4. Further investigation is clearly needed to identify the target kinase site(s) required for full NCKX4 stimulation.

**DISCUSSION**

In the present study, we examined the relationship between purinergic signalling and NCKX4 activity from a mechanistic perspective. We demonstrated through our functional assay using NCKX4-transfected HEK-293 cells that NCKX4 activity can be stimulated robustly with the purinergic agonist ATP. Based on pharmacological intervention and mutagenesis data, we propose a coincidence detection model in which the conditions for NCKX4 stimulation can only be fulfilled by the simultaneous activation of PKC and CaMKII. Purinergic agonists achieve this by activating P2Y receptors coupled via Gq/11 protein to the activation of PLCγ, leading to the production of IP3 and DAG. IP3-triggered release of Ca2+ from the ER (endoplasmic reticulum) then binds to (i) calmodulin, which activates CaMKII, and (ii) PKC, which causes the kinase to translocate to the plasma membrane for subsequent activation by DAG. While our data support the P2Y receptor pathway as the predominant route for NCKX4 stimulation, the lack of a highly specific P2Y receptor antagonist and the ambiguous data using high concentrations of α,β-methylene ATP (Figure 4) prevented us from completely ruling out the involvement of the P2X receptor. Intriguingly, it appears that the Ca2+ which enters through NCKX4 during the induced bursts of reverse-mode operation is not sufficient, either alone or together with PMA treatment, to induce stimulation of NCKX4 activity, whereas Ca2+ release from the ER via Tg is sufficient (Figure 6). This suggests that Ca2+ released from the ER has preferential access to activation of CaMKII and/or PKC isoforms compared to Ca2+ entering across the plasma membrane, perhaps due to subcellular localization of these enzymes. This source-dependency for the effect of Ca2+ also argues against a role for P2X-mediated Ca2+ entry in the activation of NCKX4.

The dual requirement for both PKC and CaMKII activation to stimulate NCKX4 provides an explanation for why PMA treatment alone is insufficient for stimulation. However, despite previous studies to the contrary [31,32], it remains possible that PMA did not result in activation of the ‘classical’ PKC isoforms necessary for NCKX4 stimulation. To test the involvement of ‘classical’ PKC isoforms we used the selective inhibitor, Go6983, which prevented NCKX4 stimulation by either ATP or Tg + PMA.
NCKX4 stimulation requires dual activation of PKC and CaMKII

Figure 8 ATP stimulation of NCKX4 mutants
HEK-293 cells were transfected with cDNA encoding either WT (wild-type) mouse NCKX4 or NCKX4 containing the indicated single, double or triple mutants. These cells were stimulated with 0.2 mM ATP, added at the arrow, and analysed as described in Figure 2. Two-way ANOVA analysis was used to test for statistical differences between normalized rates for mutants or treatments and pulses. (A) A cartoon topology model for NCKX4 illustrating the location of putative phosphorylation sites mutated in this study (T256, T312 and S325; the amino acid context of these residues is noted below the cartoon). (B) WT-control, n = 4; T256A-control, n = 2; WT + ATP, n = 7; T256A + ATP, n = 7. There was no statistically significant difference between WT + ATP and T256A + ATP. (C) WT-control, n = 3; T312A-control, n = 2; WT + ATP, n = 9; T312A + ATP, n = 17. The normalized rates for WT + ATP and T312A + ATP were statistically different (P < 0.001; 0.01; 0.01 for pulses 4–6, respectively). (D) WT-control, n = 3; S325A-control, n = 2; WT + ATP, n = 3; S325A + ATP, n = 5. There was no statistically significant difference between WT + ATP and S325A + ATP. (E) WT-control, n = 3; T256A/T312A-control, n = 2; WT + ATP, n = 10; T256A/T312A + ATP, n = 12. The normalized rates for WT + ATP and T256A/T312A + ATP were statistically different (P < 0.001; 0.001; 0.01 for pulses 4–6, respectively). (F) WT-control, n = 3; T256A/T312A/S325A-control, n = 2; WT + ATP, n = 6; T256A/T312A/S325A + ATP, n = 7. The normalized rates for WT + ATP and T256A/T312A/S325A + ATP were statistically different for pulses 4–6 (P < 0.001).

(Figure 7). While these data suggest that one or more ‘classical’ PKC isoforms are involved, further experiments would be needed to confirm this supposition.

Given the dual requirement for PKC and CaMKII activation, it is surprising that Tg alone was sufficient for partial NCKX4 stimulation. Since the combination treatment of Tg and PMA results in a level of NCKX4 stimulation higher than that induced by ATP treatment, it is possible that Tg is activating a different, additive pathway. On the other hand, the fact that Tg + PMA stimulation can be prevented by treatment with either PKC or CaMKII inhibitors, just like ATP stimulation, suggests simply a more robust activation of similar pathways. Possibly the more sustained nature of Ca2+ release induced by Tg compared with ATP results in the production of lipid mediators, via regulation...
of phospholipase A2 or phospholipase D [34,35], that allow PKC as well as CaMKII activation under these conditions.

Although we have identified two kinase systems responsible for stimulation of NCKX4 activity, the target site for their actions remains elusive. Three sites conforming to either the PKC or CaMKII consensus and located in predicted cytoplasmic loops were tested by mutagenesis. Of these, only one (T312A) had a partial inhibitory effect on ATP-induced stimulation. It is possible that additional Ser or Thr residues that conform to a weaker consensus are required in addition to T312, and can account for kinase stimulation by direct phosphorylation of the NCKX4 protein. Alternatively, it is possible that other molecule(s) lying downstream from PKC and CaMKII are required for NCKX4 stimulation. Using selective pharmacological inhibition, we tested for the possible involvement of other kinases in the MEK [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase)], AMPK (AMP-activated protein kinase) and Akt (protein kinase B) families. None of these treatments, however, influenced ATP-induced stimulation of NCKX4 (results not shown).

The experiments in this paper all used ionic modulation to induce reverse-mode Ca\(^{2+}\) entry to measure NCKX4 activity. This approach allows the isolation of NCKX activity from other plasma membrane or intracellular Ca\(^{2+}\) transport pathways. Since our data indicate an effect of stimulation on the maximal activity of the exchanger rather than individual ion-binding site affinities, it seems likely that purinergic stimulation will also influence the more physiologically relevant forward-mode, Ca\(^{2+}\) extrusion activity of the exchanger. Similar reasoning has been used when examining Ca\(^{2+}\)-regulation of the cardiac exchanger, NCX1 [36].

Synaptic plasticity is crucially dependent on Ca\(^{2+}\)-mediated kinase pathways modulating the activity of plasma membrane ion channels. Much work has demonstrated important roles for both PKC and CaMKII in the regulation of activity and membrane insertion of AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid)-receptor ion channels [37–39]. However, relatively little has been reported previously concerning the integrated regulation of Ca\(^{2+}\) homoeostasis that also requires control of Ca\(^{2+}\) efflux pathways. Here we have demonstrated that NCKX4 activity, a principle component of Ca\(^{2+}\) extrusion in brain neurons, is stimulated only by dual activation of PKC and CaMKII pathways. Intriguingly, the close family member, NCKX2, is stimulated similarly, but required only activation of PKC [15]. Thus, the precise control of Ca\(^{2+}\) homoeostasis depends on the cellular expression and subcellular localization of both transporter isoforms and regulatory kinase signalling pathways. The regulated activity of NCKX-mediated Ca\(^{2+}\) extrusion, integrated with Ca\(^{2+}\) entry pathways, is therefore critical to understanding molecular pathways of synaptic plasticity.

It remains unclear if NCKX4 stimulation corresponds to an allosteric increase in the activity of existing exchanger units in the plasma membrane, the recruitment of new units from a submembrane reservoir or both. In the context of Ca\(^{2+}\)-mediated synaptic plasticity, regulation of the AMPA-receptor offers a potentially interesting comparison. In this case, stimulation of ion channel activity is mediated largely by recruitment of AMPA-receptor channels from an intracellular storage compartment to the post-synaptic membrane. Redistribution in response to synaptic Ca\(^{2+}\) signals requires the synergistic action of both CaMKII and PKC [40–42]. In addition, mutation of the key CaMKII site, S831 in the GluR1 subunit, reduced the magnitude of AMPA current stimulation, but did not prevent it [41,43]. These intriguing parallels between regulation of NCKX4 and the AMPA receptor clearly warrant further investigation.

In summary, our current study reveals a purinergic-dependent stimulation of NCKX4 that requires the simultaneous presence and activation of PKC and CaMKII. While the effect of stimulation can be reduced by mutating a putative PKC phosphorylation site T312, the specific mechanisms still require detailed investigation. At present, our data provide the first evidence of NCKX4 regulation in a physiologically relevant context, and open up the door to understanding the complex regulatory and physiological properties of this protein.

### ACKNOWLEDGEMENTS

We thank Drs Andrew Braun and Michael Walsh (University of Calgary), and members of the Lytton laboratory for helpful discussions and useful comments.

### AUTHOR CONTRIBUTION

Jonathan Lytton conceived the study and wrote the paper. Jonathan Lytton and Xu (Jack) Yang designed the experiments. Xu (Jack) Yang performed the experiments.

### FUNDING

This work was supported by an operating grant from the Canadian Institutes of Health Research [grant no. FRN 97876]. J.L. is an Alberta Innovates-Health Solutions Senior Medical Scientist and a Canada Research Chair Tier 1 chair-holder.

### REFERENCES

1. Blaustein, M. P. and Lederer, W. J. (1999) Sodium/calcium exchange: its physiological implications. Physiol. Rev. 79, 763–854
2. Clapham, D. E. (2007) Calcium signaling. Cell 131, 1047–1058
3. Kim, M. H., Lee, S. H., Park, K. H. and Ho, W. K. (2003) Distribution of K\(^{+}\)-dependent Na\(^{+}\)/Ca\(^{2+}\) exchangers in the rat suprachiasmatic suprachiasmatic nucleus. J. Neurosci. 23, 11673–11680
4. Lee, S. H., Kim, M. H., Park, K. H., Earm, Y. E. and Ho, W. K. (2003) K\(^{+}\)-dependent Na\(^{+}\)/Ca\(^{2+}\) exchange is a major Ca\(^{2+}\) clearance mechanism in axon terminals of rat neurophysiophic neurons. J. Neurosci. 20, 891–899
5. Lytton, J. (2007) Na\(^{+}\)/Ca\(^{2+}\) exchangers: three mammalian gene families control Ca\(^{2+}\) transport. Biochem. J. 406, 355–382
6. Cai, X. and Lytton, J. (2004) The cation/Ca\(^{2+}\) exchanger superfamily: phylogenetic analysis and structural implications. Mol. Biol. Evol. 21, 1692–1703
7. Emery, L., Whelan, S., Hirschi, K. D. and Pittman, J. K. (2012) Protein Phylogenetic Analysis of Ca\(^{2+}\)/cation antiporers and insights into their evolution in plants. Front Plant Sci. 3, 1
NCKX4 stimulation requires dual activation of PKC and CaMKII

8 Altimimi, H. F. and Schnetkamp, P. P. (2007) Na\(^+\)/Ca\(^2+\) exchangers (NCKX): functional properties and physiological roles. Channels (Austin) 1, 62–69

9 Quednau, B. D., Nicoll, D. A. and Philipson, K. D. (2004) The sodium/calcium exchanger family-SLC8. Pflugers Arch. 447, 543–548

10 Liao, J., Li, H., Zeng, W., Sauer, D. B., Belmares, R. and Jiang, Y. (2012) Structural insight into the ion-exchange mechanism of the sodium/calcium exchanger. Science 335, 866–869

11 Lytton, J., Li, X. F., Dong, H. and Kraev, A. (2002) K\(^+\)-dependent Na\(^+\)/Ca\(^2+\) exchangers in the brain. Ann. N. Y. Acad. Sci. 976, 382–393

12 Stephan, A. B., Tobochnik, S., Dibattista, M., Wall, C. M., Reisert, J. and Zhao, H. (2012) The Na\(^+\)/Ca\(^2+\) exchanger NCKX4 governs termination and adaptation of the mammalian olfactory response. Nat. Neurosci. 15, 131–137

13 Parry, D. A., Poultet, J. A., Logan, C. V., Brooksie, S. J., Jafri, H., Ferguson, C. H., Anwari, B. M., Rashid, Y., Zhao, H., Johnson, C. A. et al. (2013) Identification of mutations in SLC24A4, encoding a potassium-dependent sodium/calcium exchanger, as a cause of Amelogenesis Imperfecta. Am. J. Hum. Genet. 92, 307–312

14 Altimimi, H. F. and Schnetkamp, P. P. (2007) Na\(^+\)-dependent inactivation of the retinal cone/brain Na\(^+\)/Ca\(^2+\) exchanger NCKX2. J. Biol. Chem. 282, 3720–3729

15 Lee, J. Y., Visser, F., Lee, J. S., Lee, K. H., Soh, J. W., Ho, W. K., Lytton, J. and Lee, S. H. (2006) Protein kinase C-dependent enhancement of activity of rat brain NCKX2 heterologously expressed in HEK293 cells. J. Biol. Chem. 281, 39205–39216

16 Burnstock, G. (2007) Physiology and pathophysiology of purinergic neurotransmission. Physiol. Rev. 87, 659–797

17 Abbracchio, M. P., Burnstock, G., Verkhratsky, A. and Zimmermann, H. (2009) Purinergic signalling in the nervous system: an overview. Trends Neurosci. 32, 19–29

18 Verkhratsky, A., Orkand, R. K. and Kettenmann, H. (1998) Glial calcium: homeostasis and signaling function. Physiol. Rev. 78, 99–141

19 Burnstock, G. (2008) Purinergic signalling and disorders of the central nervous system. Nat. Rev. Drug Discov. 7, 575–590

20 Abbracchio, M. P., Burnstock, G., Boeynaems, J. M., Barnard, E. A., Boyer, J. L., Kennedy, C., Knight, G. E., Fumagalli, M., Gachet, C., Jacobson, K. A. and Weisman, G. A. (2006) International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol. Rev. 58, 281–341

21 Hiruma, H. and Bourque, C. W. (1995) P2 urinopenor-mediated depolarization of rat supraoptic neurosecretory cells in vitro. J. Physiol. 489 (Pt 3), 805–811

22 Jo, Y. H. and Schlichter, R. (1999) Synaptic corelease of ATP and GABA in cultured spinal neurons. Nat. Neurosci. 2, 241–245

23 Saucerman, J. J., Brunton, L. L., Michailova, A. P. and McCulloch, A. D. (2003) Modeling beta-adrenergic control of cardiac myocyte contractility in silico. J. Biol. Chem. 278, 47997–48003

24 Yousuf, A., Klinger, F., Schicker, K. and Boehm, S. (2011) Nucleotides control the excitability of sensory neurons via two P2Y receptors and a bifurcated signaling cascade. Pain 152, 1809–1901

25 Li, X. F., Kiedrowski, L., Tremblay, F., Fernandez, F. R., Perizzolo, M., Winkfein, R. J., Turner, R. W., Bains, J. S., Rancourt, D. E. and Lytton, J. (2006) Importance of K\(^+\)-dependent Na\(^+\)/Ca\(^2+\) exchanger 2, NCKX2, in motor learning and memory. J. Biol. Chem. 281, 6273–6282

26 Kim, M. H., Korogod, N., Schneggenburger, R., Ho, W. K. and Lee, S. H. (2005) Interplay between Na\(^+\)/Ca\(^2+\) exchangers and mitochondria in Ca\(^2+\) clearance at the calyx of Held. J. Neurosci. 25, 6057–6065

27 Visscher, F., Valsecchi, V., Annunziato, L. and Lytton, J. (2007) Analysis of ion interactions with the K\(^+\)-dependent Na\(^+\)/Ca\(^2+\) Exchangers NCKX2, NCKX3, and NCKX4: identification of Thr-551 as a key residue in defining the apparent K\(^+\) affinity of NCKX2. J. Biol. Chem. 282, 4453–4462

28 Velazquez, B., Garrad, R. C., Weisman, G. A. and Gonzalez, F. A. (2000) Differential agonist-induced desensitization of P2Y2 nucleotide receptors by ATP and UTP. Mol. Cell Biochem. 206, 75–89

29 White, P. J., Webb, T. E. and Boarder, M. R. (2003) Characterization of a Ca\(^2+\) response to both ATP and ATP at human P2Y11 receptors: evidence for agonist-specific signaling. Mol. Pharmacol. 63, 1356–1363

30 Burnstock, G. and Kennedy, C. (1985) Is there a basis for distinguishing two types of P2-purinoceptor? Gen Pharmacol. 16, 433–440

31 Nishizuka, Y. (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334, 661–665

32 Newton, A. C. (1997) Regulation of protein kinase C. Curr. Opin. Cell Biol. 9, 161–167

33 Lytton, J., Westlin, M. and Hanley, M. R. (1991) Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. J. Biol. Chem. 266, 17067–17071

34 Leslie, C. C. (1997) Properties and regulation of cytosolic phospholipase A2. J. Biol. Chem. 272, 16709–16712

35 Exton, J. H. (1999) Regulation of phospholipase D. Biochim. Biophys. Acta 1439, 121–133

36 Omelchenko, A., Bouchard, R., Shurrar, S., Trac, M., Hnatowich, M. and Hryshko, L. V. (2005) Frequency-dependent regulation of cardiac Na\(^+\)/Ca\(^{2+}\) exchanger. Am. J. Physiol. Heart Circ. Physiol. 289, H1594–H1603

37 Abeliovich, A., Chen, C., Goda, Y., Silva, A. J., Stevens, C. F. and Tonegawa, S. (1993) Modified hippocampal long-term potentiation in PKC gamma-mutant mice. Cell 75, 1253–1262

38 Silva, A. J., Paylor, R., Wehner, J. M. and Tonegawa, S. (1992) Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. Science 257, 206–211

39 Kessels, H. W. and Malinow, R. (2009) Synaptic AMPA receptor plasticity and behavior. Neuron 61, 340–350

40 Barria, A., Derkach, V. and Soderling, T. (1997) Identification of the Ca\(^2+\)/calmodulin-dependent protein kinase II regulatory phosphorylation site in the alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionate-type glutamate receptor. J. Biol. Chem. 272, 32727–32730

41 Lee, H. K., Takamiya, K., Han, J. S., Man, H., Kim, C. H., Rumbaugh, G., Yu, S., Ding, L., He, C., Petralia, R. S. et al. (2003) Phosphorylation of the AMPA receptor GluR1 subunit is required for synaptic plasticity and retention of spatial memory. Cell 112, 631–643

42 Boehm, J., Kang, M. G., Johnson, R. C., Esteban, J., Huganir, R. L. and Malinow, R. (2006) Synaptic incorporation of AMPA receptors during LTP is controlled by a PKC phosphorylation site on GluR1. Neuron 51, 213–225

43 Hayashi, Y., Shi, S. H., Esteban, J. A., Piccini, A., Poncer, J. C. and Malinow, R. (2000) Driving AMPA receptors into synapses by LTP and CaMKII: requirement for GluR1 and PDZ domain interaction. Science 287, 2262–2267