

Coregulation of Multiple Signaling Mechanisms in pp60v-Src-Induced Closure of Cx43 Gap Junction Channels

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Abstract Attenuation in gap junctional coupling has consistently been associated with induction of rapid or synchronous cell division in normal and pathological conditions. In the case of the v-src oncogene, gating of Cx43 gap junction channels has been linked to both direct phosphorylation of tyrosines (Y247 and 265) and phosphorylation of the serine targets of Erk1/2 (S255, 279 and 282) on the cytoplasmic C-terminal domain of Cx43. However, only the latter has been associated with acute, rather than chronic, gating of the channels immediately after v-src expression, a process that is mediated through a “ball-and-chain” mechanism. In this study we show that, while ERK1/2 is necessary for acute closure of gap junction channels, it is not sufficient. Rather, multiple pathways converge to regulate Cx43 coupling in response to expression of v-src, including parallel signaling through PKC and MEK1/2, with additional positive and negative regulatory effects mediated by PI3 kinase, distinguished by the involvement of Akt.

Keywords Cx43 · Gap junction · v-Src · Erk1/2 · Signal transduction

Introduction

Mammalian gap junctions, composed of membrane proteins called connexins, represent arrays of transmembrane channels that allow low-molecular weight molecules and ions to move directly between the cytoplasm of opposed cells (Goldberg et al. 1998; Kumar and Gilula 1996). Reduced communication through gap junction channels has been frequently correlated with increases in cell proliferation of both normal and transformed cells (Berthoud et al. 1993; Cronier et al. 2009). An early response to the stimulation of many cells by growth factors in culture is a decrease in coupling just prior to initiation of mitosis (Lau et al. 1992). Similarly, several oncogenes have been shown to reduce coupling of cells, most notably pp60^{v-src}, which Ross Johnson first observed 31 years ago to acutely induce closure of gap junction channels (Atkinson et al. 1981). It has been proposed that this uncoupling could serve to isolate cells from the inhibitory signals of neighboring cells and/or enable the accumulation of positive stimuli within the cells that generate them (Loewenstein 1990). In the current work, we return to a more detailed analysis of the signals that link v-src expression to the closure of Cx43 gap junction channels, to compare the similarities and differences of the oncogenic process to that induced by normal growth factor signaling.

Several growth factors [EGF (Lau et al. 1992), PDGF (Hossain et al. 1998, 1999b), and insulin-like growth factor (Homma et al. 1998)] have been shown to cause acute closure of Cx43 gap junction channels, primarily associated with activation of ERK, and its direct phosphorylation of Cx43

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(Kanemitsu and Lau 1993; Hossain et al. 1998). However, other kinase pathways, such as PKC (Hossain et al. 1998, 1999a, b), have also been implicated. Several phosphorylation sites for these and other kinases have been mapped on the C-terminal domain of Cx43 (Berthoud et al. 1993; Kanemitsu et al. 1998; Warn-Cramer et al. 1996; reviewed in Lampe and Lau 2004). Consistent with this, removal of the C-terminal domain was shown to ablate gating by insulin-like growth factor, which could then be rescued by addition of the C-terminal domain as a separate peptide (Homma et al. 1998). This led to the model that this phosphorylation-driven gating in response to growth factors occurs by a “ball-and-chain” mechanism, similar to what had been shown for pH gating of Cx43 (Ek-Vitorin et al. 1996; Morley et al. 1996) and much earlier for inactivation of K^+ channels (Hoshi et al. 1990). However, while these studies had focused on acute gating of the channels, there is also evidence that growth factors can more chronically induce internalization of gap junction structures over longer periods of time. Hence, the interaction of the cytoplasmic domain of Cx43 with various signaling [e.g., src via SH2 and 3 domains (Warn-Cramer et al. 1996)], adapter [e.g., ZO-1 via PDZ 1 (Giepmans and Moolenaar 1998; Toyofuku et al. 1998), and 14-3-3 protein (Park et al. 2007)] and cytoskeletal [e.g., tubulin (Giepmans et al. 2001)] elements may also be relevant to longer-term regulation of coupling by growth factors.

While the regulation of gap junctions by v-src was first studied 10 years before that with growth factors, the precise mechanism of how v-src induces loss of coupling either acutely or chronically has remained somewhat controversial. By analogy with the closure of Cx43 channels by growth factors described above, Zhou et al. (1999) demonstrated that v-src closure of Cx43 channels depended not on the direct src targets on Cx43 [Y265 and 247 (Swenson et al. 1990; Solan and Lampe 2008)] but on the presence of three ERK1/2 phosphorylation sites (S255, 279 and 282) mapped in the C-terminal domain by Warn-Cramer et al. (1996). The dependence on ERK activity was also demonstrated pharmacologically in normal rat kidney (NRK) cells expressing a temperature-sensitive (ts) pp60v-src. Consistent with this, Ito et al. (2006) implicated the Ras-Raf pathway, which directly activates ERK, in the gating of Cx43 gap junction channels by v-src. Others have also implicated Cas as being essential for src gating, although the mechanism of this effect remains unclear (Shen et al. 2007). Finally, truncations of the C-terminal domain led to a loss of response of Cx43 to v-src, which was restored by coexpression of the C-terminal domain as an independent polypeptide, consistent with the “ball and chain” mechanism implicated in growth factor gating of Cx43.

However, contrary results have been published by Swenson et al. (1990) and Lin et al. (2001), who showed that Y265 and Y247 are required for v-src-induced disruption of

coupling in both *Xenopus* oocytes (Swenson et al. 1990) and a mouse Cx43 knockout cell line transfected with different Cx43 mutants (Lin et al. 2001). In the latter study, the ERK1/2 phosphorylation sites (S255, 279 and 282) were found not to be required for closure. The differences between these seemingly contradictory findings do not correlate with the expression system as both results have been reported in oocytes and mammalian cells. However, one consistent distinction is that ERK dependence was reported when src's effect was exerted on preformed Cx43 channels and was likely associated with initial gating immediately following v-src expression. This is likely to be similar to the transient closing of Cx43 channels in response to cytokines [PDGF (Hossain et al. 1998) and EGF (Kanemitsu and Lau 1993)]. By contrast, tyrosines were implicated when v-src was expressed prior to, or concurrently with, Cx43 and could represent a more chronic mechanism for closure of Cx43 channels that diverges from known growth factor pathways. Using phosphorylation state-specific antibodies, Solan and Lampe (2008) showed that, in LA-25 cells, tyrosine phosphorylation predominantly occurred in gap junction plaques when v-src was activated. They also showed increased phosphorylation of ERK and PKC sites in Cx43 upon v-src activation, suggesting a role of multiple signaling pathways in gap junction downregulation during src transformation. However, they were unable to address the issue of whether these phosphorylation sites were functionally required for gap junction closure, nor could they assess the timeline of these phosphorylation events following src expression. In most cell lines this poses a problem, unless src expression can be acutely activated, such as in ts mutants. Unfortunately, the better-characterized ts v-src constructs have often proven to be unstable.

An alternative model system is the *Xenopus* oocyte, where src can be acutely activated by injection of its encoding RNA, allowing the time course of the response to be followed. Most mitogenic signaling cascades are present and well characterized in *Xenopus* oocytes, and they have been used extensively in the electrophysiological characterization of gap junction channels. In this study, we employed this expression system to conduct a comprehensive analysis of the regulatory pathways that mediate the initial gating of Cx43 channels by v-src to explore the degree to which they may use similar or distinct pathways from those implicated in growth factor-mediated Cx43 gating.

Materials and Methods

cDNA Constructs

Rat Cx43 cDNA (provided by Dr. Eric Beyer, University of Chicago, Chicago, IL) was subcloned into the PGEM-7Zf⁺

vector (Promega, Madison, WI) at the *EcoRI* site. All mutants were provided by Drs. Steve Taffet and Mario Delmar (State University of New York Health Science Center, Syracuse, NY). The cDNA for pp60v-src was provided by Dr. Marilyn Resh (Memorial Sloan-Kettering Cancer Center, New York, NY). The cDNA for constitutively active MEK1 (human CA-MEK1), a kind gift from Dr. Natalie Ahn (University of Colorado, Boulder, CO), was subcloned into the oocyte expression vector pBlue-script MXT at the *EcoRV* and *XbaI* restriction sites. Constitutively active and dominant negative (DN) PKC (PKC α/ϵ) constructs (murine) were provided by Dr. Elissavet Kardami (University of Manitoba, Winnipeg, Canada) in the pSVK3 vector.

Preparation of cRNAs

All cRNAs were linearized and transcribed in vitro using Ampliscribe Transcription kits (Epicentre, Madison, WI) according to the manufacturer's recommendations. The resultant cRNAs were quantitated after DNase-1 (Sigma-Aldrich, St. Louis, MO) treatment by absorbance at 260 nm.

Xenopus Oocyte Expression System and Measurement of Junctional Conductance

Oocytes were unilaterally extracted from female *Xenopus laevis* toads and treated with 1 mg/ml collagenase (Sigma-Aldrich) to digest most of the follicular layer. Oocytes were preinjected with 40 nl of 0.2 $\mu\text{g}/\mu\text{l}$ of an oligonucleotide complementary to *Xenopus* Cx38, 5'-75 GCTTTA GTAATTCCCATCCTGCCATGTTTC 45-3', prior to injection with Cx43 cRNA (2 ng/oocyte) as described by Zhou et al. (1999). After final manual stripping of the vitelline membrane, oocytes were paired for ~16 h prior to measuring junctional currents (I_j) by dual-cell voltage clamp as described in Zhou et al. (1999). All experiments were carried out in oocyte batches from at least five different females, to account for differences in batch-specific physiological behavior.

Measure of Kinase Effects

About 16 h after pairing, oocytes were recorded for approximately 20 min to ensure stable conductance levels before secondary injection of cRNAs for the kinase of interest [i.e., v-src (8 ng), CA-PKC (8 ng) or CA-MEK (8 ng)]. The effects of secondary injection on gap junctional conductance were assessed after 6 h and expressed as fractional decrements of the conductance recorded from the same oocyte pair before introduction of the kinase cRNA. Thus, the effects of kinases were normalized within the

same oocyte. In cases where DN constructs were used, the cRNAs encoding DN-PKA α (8 ng) and DN-PKC ϵ (8 ng) were injected at the same time as v-src cRNA injection. Expression of each construct was tested by Western blot analysis in each batch of oocytes as described below.

Pharmacological Inhibitors

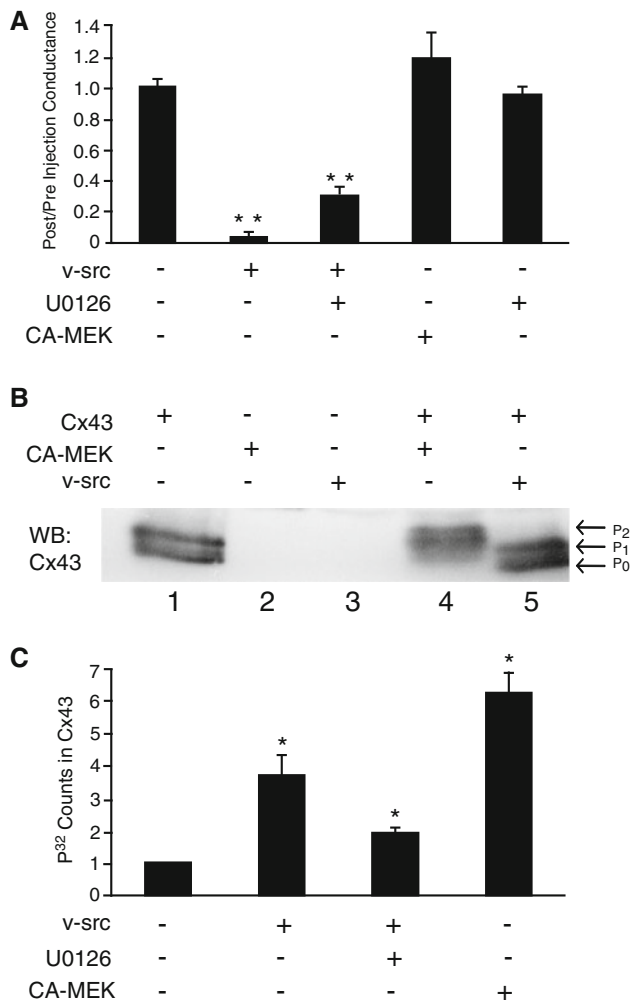
Bis(indolyl-1)maleamide (BIM; Calbiochem, La Jolla, CA) was added to 0.1 μM final concentration, U0126 and LY294002 (Cell Signaling technology, Beverly, MA) were added to 50 μM final concentration, wortmannin (Sigma-Aldrich) was added to 0.05 μM final concentration and Akt inhibitor VIII (Akti-1/2, Calbiochem) was added to 10 μM final concentration. All inhibitors were added to L-15 *Xenopus* oocyte incubation medium (Sigma-Aldrich) just before injection of the cRNA for pp60v-src, CA-MEK1/2 or CA-PKC ϵ . The medium with the relevant inhibitor was changed every hour to ensure its full effect over the entire duration of the experiment.

Immunoprecipitation and Western Blot

Oocyte lysates were prepared from approximately 15–20 oocytes, injected with the same schedule as used in the functional assays. Lysates were prepared in modified RIPA buffer [1 % NP-40, 0.1 % SDS, 50 ml Tris (pH 7.4), 100 mM NaCl, 2 mM EDTA, 50 mM NaF, 40 mM β -glycerophosphate, 1 mM Na₂VO₄ and protease inhibitors]. The supernatant was collected from clarified lysates and immunoprecipitated with anti-Cx43 polyclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Erk1/2 polyclonal antibody (1:500; Upstate Biotechnology, Lake Placid, NY) overnight at 4 °C. For Western blot analysis of oocyte lysates, oocytes were lysed in 200 μl of RIPA buffer and clarified at 14,000 rpm for 30 min. Lysates were resolved on a 10 % SDS-polyacrylamide gel before transfer onto a PVDF membrane (Millipore, Billerica, MA). Since *Xenopus* oocytes express only Erk2 (Ferrell 1999), a single band is observed when probed for Erk1/2 or phospho-Erk1/2. For Cx43 detection, anti-Cx43 monoclonal antibody (Chemicon, Temecula, CA) targeted to the C-terminal tail of Cx43 was used as the primary antibody. To test for expression of kinase constructs, anti-avian Src antibody (clone EC10, 1:1,000; Upstate Biotechnology), anti-PKC ϵ (Cell Signaling Technologies) and anti-PKC ϵ mouse monoclonal antibody (1:1,000, Santa Cruz Biotechnology) were used.

Radiolabeled Assay for Cx43 Phosphorylation

To assay for MEK-induced Cx43 phosphorylation, ATP- γ 32 (2–10 $\mu\text{Ci}/\text{oocyte}$, 250 $\mu\text{Ci}/\mu\text{l}$; Amersham Biosciences,



Arlington Heights, IL) was injected along with CA-MEK1 mRNA into Cx43 coupled oocytes. After 6 h, oocytes were lysed as described in Zhou et al. (1999). Briefly, for each experiment, approximately six labeled oocytes were homogenized in 200 μ l/oocyte of modified RIPA buffer. The homogenate was brought to 2 % Triton X-100 after boiling for 5 min and cleared in a microcentrifuge at 13,000 rpm for 5 min. One microliter of primary antibody/oocyte (crude rabbit antisera against Cx43 residues 302–319) was added to the supernatant and pulled down by preswollen protein A-Sepharose CL-4B beads (Sigma-Aldrich). The beads were washed three times in the same RIPA buffer used for oocyte lysis, before solubilization of the immunoprecipitated material by boiling for 10 min in 2 \times SDS sample buffer and subsequent separation by SDS-PAGE on a 10 % gel. The dried gel was analyzed by exposure to a PhosphoImaging cassette (model 425E using ImageQuant v.4.2 software; Molecular Dynamics, Sunnyvale, CA) for several hours, and then bands were quantitated after reading on a PhosphoImager.

Fig. 1 a ERK1/2 is necessary, but not sufficient, for v-src gating of Cx43. Cx43 paired oocytes were injected with water, v-src or CA-MEK1; and conductance 6 h postinjection was measured as a ratio of the preinjection conductance. v-src induced almost complete inhibition of coupling, which could be partially prevented by inhibition of MEK1/2 with U0126. However, CA-MEK1 injection alone had no significant effect on Cx43 coupling. U0126 alone had no effect on coupling. The data represent means of six separate experiments, each having a minimum of eight coupled oocyte pairs. Statistical analysis was performed using Student's *t* test, comparing each group to the Cx43-expressing oocytes (***P* < 0.001). In this and all subsequent graphs, bars represent mean values, with standard errors indicated. **b** CA-MEK induces phosphorylation of Cx43. Cx43 coupled oocytes, subjected to various treatments as indicated, were lysed and then immunoprecipitated with anti-Cx43 polyclonal antibody, followed by analysis via western blot probed with a Cx43 monoclonal antibody. No bands were evident in the absence of Cx43 injection (lanes 2 and 3). A doublet corresponding to P₀ and P₁, a phosphorylated form, was found in cells injected with only Cx43 (lane 1). CA-MEK induced retardation in electrophoretic mobility of Cx43, producing a more highly phosphorylated P₂ form (lane 4). In contrast, v-src (lane 5) failed to induce a significant shift in mobility. **c** Src phosphorylation of Cx43 occurs partially through ERK but with low efficiency. Cx43 coupled oocytes were coinjected with ATP- γ -³²P and either v-src or CA-MEK1 cRNA. After 6 h of incubation, oocyte pairs were lysed, immunoprecipitated with anti-Cx43 antibody and separated by SDS-PAGE. Incorporated ³²P was quantitated by phosphoimage analysis. Src induced phosphorylation, which was about 50 % inhibited by the MEK inhibitor U0126. CA-MEK induced significantly higher phosphorylation levels than src. Data represent the mean \pm SE from three separate experiments, with equal numbers of oocyte pairs in each set. Data were normalized and compared statistically to oocytes expressing only Cx43; statistical significance was determined by Student's *t* test (**P* < 0.05)

Results

ERK1/2 is Necessary in v-src-Induced Acute Closure of Cx43 Gap Junctions

Oocytes have proven to be an effective system for analysis of acute gating of Cx43 channels by src, as the v-src protein can be expressed after Cx43 gap junction channels are stably established. This is in contrast to most mammalian cell studies where src is expressed concurrently with Cx43 and can affect many processes including assembly and degradation. We had previously shown a requirement for ERK phosphorylation of Cx43 for acute gating by src in oocytes. In mammalian cells, inhibition of both ERK (Zhou et al. 1999) and Ras (Ito et al. 2006) largely prevents src gating of Cx43, a result we now tested in oocytes. In the presence of the highly specific inhibitor of MEK1/2 activation, U0126 (Davies et al. 2000), we observed only a 2.5-fold drop in conductance of Cx43 coupled oocytes (Fig. 1a). This was slightly greater than the nonspecific effect of v-src on Cx43 with the ERK targets deleted, or on Cx32, which has no endogenous src or ERK kinase targets, and dramatically less than the 150-fold drop induced by v-src alone on Cx43. This indicated that the ERK was

required for src gating of Cx43, consistent with the previous observation that the consensus ERK phosphorylation sites on Cx43 are required for src gating (Zhou et al. 1999).

To test if it was also sufficient, CA-MEK1, which directly activates ERK1/2, was injected into Cx43 coupled oocytes, with no significant effect on gap junction coupling (Fig. 1a). This was not due to failure of expression or activity of CA-MEK1 in the oocyte system as CA-MEK1 induced both a significantly greater mobility shift of Cx43 in SDS polyacrylamide electrophoresis [this has been correlated with serine phosphorylation (Musil et al. 1990; Lampe et al. 2000) (Fig. 1b)] and ^{32}P incorporation into Cx43 (Fig. 1c) than was caused by v-src. About 50 % of src-induced phosphorylation of Cx43 could be attributed to ERK, as it could be blocked by U0126. However, the efficiency of the signal is less than that achieved by directly activating ERK through MEK. Thus, while ERK1/2 appears to be necessary for acute closure of gap junctions by v-src, it is not sufficient. We have also shown previously that direct gating of Cx43 channels by v-src is not dependent on the tyrosine targets of v-src on Cx43 (Zhou et al. 1999). Thus, it seems reasonable to deduce that other signaling pathways are likely to be involved in acute closure of Cx43 gap junctions.

PKC is Also Required for v-src-Induced Closure of Cx43 Gap Junctions

Given previous documentation of direct effects of PKC on Cx43 coupling (Moreno et al. 1994) and its implication in uncoupling Cx43 expressing cells in response to PDGF (Hossain et al. 1998) or v-src (Solan and Lampe 2008), we tested the effects of the broad-spectrum PKC inhibitor BIM. This significantly reduced the ability of v-src to close Cx43 gap junctions, although, as in the case of the MEK1/2 inhibitor U0126, this was not complete (Fig. 2a). When added together, the MEK1/2 (U0126) and PKC (BIM) inhibitors appeared to act in an additive fashion. In fact, the rescue of coupling to two-thirds of the level before src injection is comparable to that seen in src injections of Cx32 cells (a connexin that lacks src or ERK targets). This has been interpreted as reflecting non-gap junction-specific effects of src, possibly on cell adhesion. To assess if the role of PKC in v-src gating required direct phosphorylation of Cx43, the PKC targets on Cx43 were deleted. Cx43 Δ 363-373 removes PKC phosphorylation sites that have been definitively mapped [S368, S372 (Lampe et al. 2000)], while Cx43S262A and Cx43S297A delete serines within consensus PKC ϵ phosphorylation sites (Doble et al. 2001). Neither the deletion nor site-specific mutants showed any significant effect on v-src gating of Cx43 (Fig. 2b), demonstrating that PKC must affect signaling pathways upstream of the channel itself. S297 appears to be

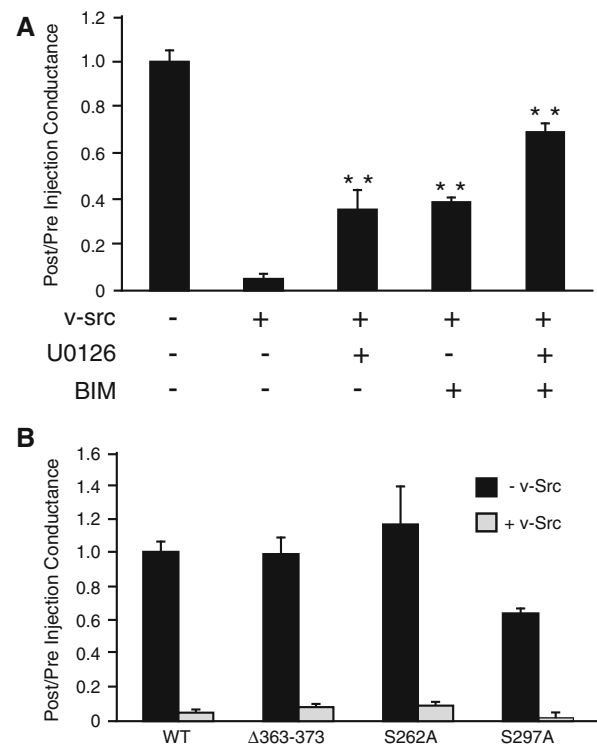


Fig. 2 **a** Both MEK1/2 and PKC play a role in v-src-induced Cx43 gating. Cx43 coupled oocytes were incubated in medium alone or in the presence of the PKC inhibitor BIM (0.1 μM), the MEK inhibitor U0126 (50 μM) or both. Individually, both U0126 and BIM decreased pp60^{v-src}-induced Cx43 closure. Combined treatment with both inhibitors showed an additive effect, indicating that MEK and PKC may act through parallel pathways. The data represent means of six separate experiments, each having a minimum of eight coupled oocyte pairs. Statistical analysis was performed by Student's *t* test, comparing each group to the Cx43 + src-injected oocytes (** $P < 0.001$). **b** PKC phosphorylation of Cx43 is not required for gating. Ablation of all documented PKC phosphorylation targets (Cx43 Δ 363-373) as well as consensus PKC ϵ phosphorylation sites on Cx43 (S362A and S297A) failed to significantly affect v-src gating of the channels. The reduced coupling in Cx43S297A cells may reflect the independent role of this site in efficient Cx43 expression. *Gray bars* represent pre-/postconductance ratios in the absence of Src, and *black bars* represent conductance ratios pre- and post-v-src cRNA injection

required for optimal function of Cx43 as Cx43S297A expressing oocytes showed consistently lower coupling, despite injection of similar cRNA levels. However, the src gating response was not impacted (Fig. 2b).

PKC has been demonstrated in other systems to be required for optimal ERK1/2 activity in response to upstream signals (Schonwasser et al. 1998). To test this in the oocyte system, we first showed that direct activation of ERK1/2 by CA-MEK1, which caused a large increase in the phospho form, was unaffected by BIM (Fig. 3, lanes 1, 2). However, ERK2 activation by the upstream effector v-src (Fig. 3, lane 3) was substantially inhibited by BIM

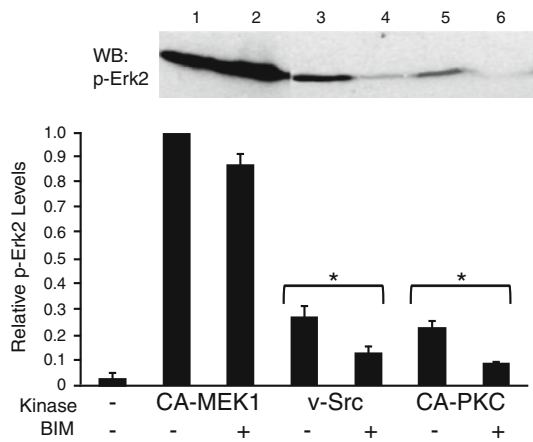


Fig. 3 ERK1/2 phosphorylation is regulated by PKC. Cx43 coupled oocytes were incubated in L-15 medium, with or without BIM (0.1 μ m); subsequently injected with cRNA for pp60^{v-src}, CA-MEK1 or CA-PKC; and immunoblotted with anti-phospho-ERK1/2 antibody (*inset*). ERK1/2 activation was evident by all three kinases (*lanes 1, 3 and 5*), although direct activation by MEK1/2 was by far the most effective. BIM did not affect ERK1/2 activation by CA-MEK1 (*lane 2*) but significantly inhibited its activation by pp60^{v-src} and CA-PKC (*lanes 4 and 6*, respectively). Data are from three separate experiments, each having equal numbers of coupled oocyte pairs for each set ($n = 10$). Statistical significance of each treatment with or without src or with or without CA-PKC was assessed by Student's *t* test ($*P < 0.05$)

(Fig. 3, lane 4). Also, ERK1/2 can be directly activated by CA-PKC (Fig. 3, lane 5), which, as might be expected, was almost completely blocked by BIM (Fig. 3, lane 6).

While BIM is an effective inhibitor of many PKC isoforms, it also can have off-target effects against other kinases like MAP kinase-activated kinase, S6 kinase, GSK3 and PI-dependent protein kinase (Davies et al. 2000). DN PKC constructs represent more specific reagents, but these are isotype-specific. The two likely candidates in this case were PKC ϵ and PKC α , since their direct phosphorylation sites on Cx43 have been identified (Doble et al. 2001; Lampe et al. 2000) and their activation by v-src is well documented (Zang et al. 1995). When DN constructs specific for each isoform were injected into oocytes, only DN-PKC α was effective, producing a similar level of inhibition of v-src gating of Cx43 as seen with BIM (Fig. 4a). Consistent with the isoform specificity of the DN constructs, only the α -isoform of CA-PKC induced partial closure of Cx43 channels (Fig. 4b, group 1). In conjunction with CA-MEK1 both isoforms had some effect on partially closing Cx43 channels, but this effect was larger for PKC α . However, the maximum reduction in coupling ($\sim 60\%$) was still far less than that induced by v-src ($>99\%$) (Fig. 4b, group 1), indicating that additional pathways still need to be considered beyond PKC.

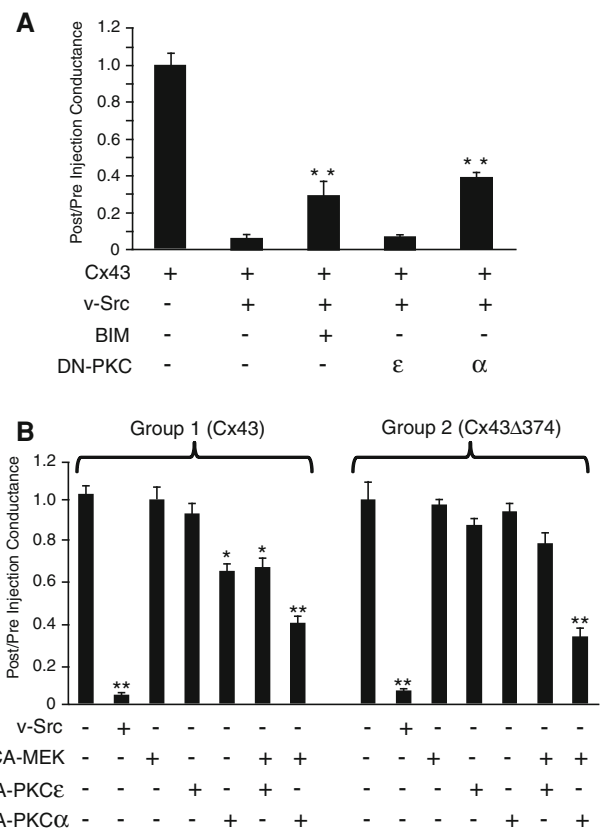


Fig. 4 a The PKC α isoform mediates v-src gating of Cx43 in oocytes. The effects of coinjection of DN isoforms of PKC α and PKC ϵ on v-src-induced Cx43 gating were compared to those of the generic PKC inhibitor BIM. DN-PKC α showed similar effects to BIM, while DN-PKC ϵ had no effect. Data represent means of six separate experiments, each having a minimum of eight coupled oocyte pairs. Statistical analysis was performed by Student's *t* test, comparing each treatment to the Cx43 + src group ($**P < 0.001$). b PKC α , in conjunction with MEK, selectively inhibits Cx43 coupling, independent of the C-terminal binding domain for ZO-1. *Group 1* CA-PKC α , but not CA-PKC ϵ , inhibited Cx43 coupling when injected into oocyte pairs. Uncoupling was enhanced by coinjection of CA-MEK, but this did not approach the degree of uncoupling generated by v-src. *Group 2* truncation of Cx43 at residue 374, removing the PDZ domain which mediates ZO-1 binding, eliminated the effect of CA-PKC α but did not change the combined MEK/PKC effect on coupling. Data represent means of six separate experiments, each having a minimum of eight coupled oocyte pairs. Statistical analysis was performed using Student's *t* test, comparing each group to oocytes expressing Cx43 only ($**P < 0.001$, $*P < 0.05$)

ZO-1 Binding Site on Cx43 Does Not Appear to Play a Role in v-src-Induced Closure of Cx43 Gap Junctions

Since v-src gating of Cx43 has been characterized as operating through a “ball-and-chain” mechanism, that would presumably require a free C terminus. Thus, we also investigated the potential role of ZO-1 binding to the C terminus as this would “tether” the tail to the cytoskeleton. This interaction, which occurs through a PDZ binding site at the C terminus of Cx43 (Giepmans and Moolenaar 1998;

Toyofuku et al. 1998), has been shown by Toyofuku et al. (2001) and Sorgen et al. (2004) to be disrupted by constitutively active c-src, invoking a possible direct role for src in gating other than through tyrosine phosphorylation. However, deletion of the ZO-1 binding site by truncation of the nine C-terminal residues (Cx43 Δ 374, a gift from Dr. M. Delmar) had no effect on the ability of v-src to close the channels and did not enhance the ability of MEK1 or CA-PKC α (separately or coordinately) to close the channel (Fig. 4b, group 2). The modest inhibition of wt Cx43 coupling observed with CA-PKC α alone (Fig. 4b, group 1) was not seen with this C-terminal truncation mutant (Fig. 4b, group 2), despite the fact that none of the consensus PKC α sites were directly eliminated.

Phosphatidylinositol-3-Kinase Plays a Complex Regulatory Role in v-src Closure of Cx43 Gap Junctions

Another potential pathway that has been implicated in src signaling is phosphatidylinositol-3-kinase (PI3K). To probe the role of this pathway in src gating of Cx43, we used two different PI3K inhibitors, reversible LY294002 and irreversible wortmannin. The former has a lower affinity and shows some cross-reactivity with casein kinase II and GSK3 β , while the latter has a higher affinity and different cross-reactivity with smooth muscle myosin light chain kinase (Davies et al. 2000), likely not relevant in the current study. Despite the different properties of these two inhibitors, both caused similar inhibition ($P < 0.05$) of v-src gating (Fig. 5, groups 2 and 3, respectively), albeit less efficiently than we had observed with either the MEK1/2 (U0126) or PKC inhibitors (BIM) (Fig. 5, group 1; $P < 0.001$). No significant increase in inhibition was seen when either wortmannin or LY294002 was used in combination with BIM compared to BIM alone. However, the application of U0126 in concert with wortmannin or LY294002 caused an unexpected restoration of v-src gating of Cx43, reversing the inhibition seen with either inhibitor alone. The application of all three inhibitors caused a similar “annulment” of their individual effects and the synergistic effects of the PKC and ERK pathways and restored full closure of Cx43 channels by v-src. None of the inhibitors, alone or in combination, affected Cx43 coupling in the absence of v-src (Fig. 5, group 4), indicating no deleterious effects on oocytes from the combination of inhibitors used.

Further insights into the apparently antagonistic effects of wortmannin and U0126 were obtained by directly examining levels of activated ERK under the different treatments. Wortmannin treatment not only enhanced the activation of ERK1/2 following v-src treatment (Fig. 6, lanes 2 and 3; $P < 0.001$) but also reversed the reduction of src-induced ERK phosphorylation caused by U0126 (Fig. 6, lanes 4 and

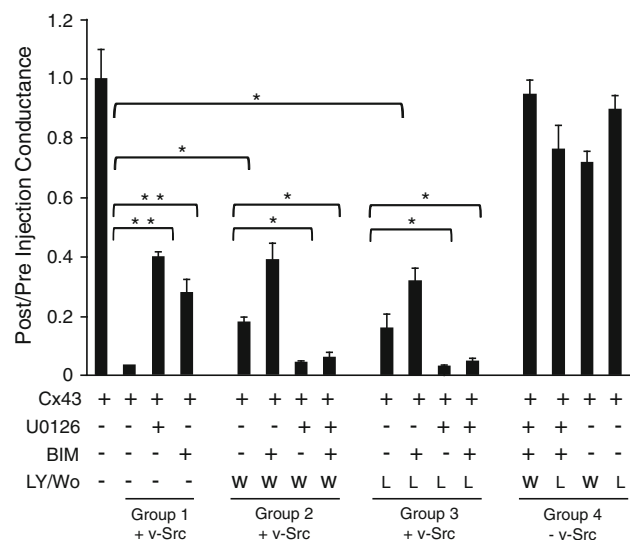


Fig. 5 PI3K plays a complex role in v-src-induced gating. Inhibition of PI3K by wortmannin or LY294002 partially blocked the ability of v-src to close Cx43 channels (compare first bars in group 2 or 3, respectively, with second bar in group 1). When added together with BIM, both wortmannin and LY294002 slightly enhanced the inhibition of v-src gating (second bars, groups 2 and 3), although this was significant only in the case of wortmannin. However, when wortmannin or LY294002 was added in conjunction with U0126, with or without BIM (third and fourth bars, groups 2 and 3), the effects of both inhibitors appeared to be annulled, restoring full closure of Cx43 by v-src. None of the inhibitors, alone or in combination, had a significant effect on Cx43 coupling in the absence of v-src (group 4). Data represent the mean \pm SE from three separate experiments with an equal number of oocyte pairs in each set. Brackets indicate specific comparisons subjected to Student's *t* test, yielding differences at the $**P < 0.001$ or the $*P < 0.05$ level

5; $P < 0.05$). Thus, PI3K may play dual roles in the src gating of Cx43, facilitating the process, possibly through PKC, while also exerting an inhibitory influence through effects on ERK activation. In an effort to distinguish which aspects of the diverse PI3K pathways might mediate these apparently opposing effects on v-src gating, we tested the role of the major PI3K effector Akt. Akti, a highly specific inhibitor of both Akt isoforms 1 and 2 (Barrett et al. 2005), caused a similar level of inhibition of v-src gating as had the PI3K inhibitor LY294002 (Fig. 7). However, unlike the PI3K inhibitors, application of Akti in conjunction with the ERK inhibitor U0126 caused no restoration of v-src gating (Fig. 7). Thus, it appears that PI3K's role in contributing to v-src-induced closure of Cx43 channels is mediated by Akt, while its positive effects on Cx43 coupling through antagonism of the ERK pathway do not involve Akt.

Discussion

The mechanisms for inhibition of cell coupling during transformation remain unclear. Chronic uncoupling in

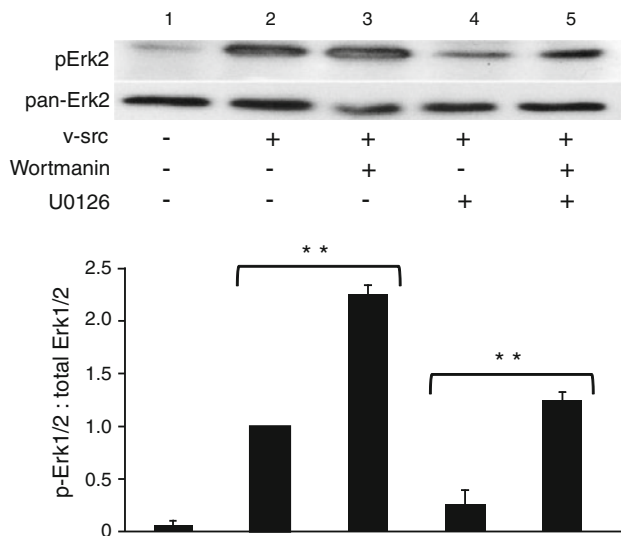


Fig. 6 PI3K activity inhibits phosphorylation of ERK1/2. Immunoblot, using anti-phospho ERK1/2 (*upper panel*) and pan-specific ERK1/2 (*lower panel*) antibodies, of lysates from Cx43 coupled, v-src injected cells (*lanes 2–5*) treated with either wortmannin (*lane 3*), U0126 (*lane 4*) or both (*lane 5*). In either the presence or the absence of the MEK1/2 inhibitor, wortmannin induced an increase in ERK phosphorylation. There was no significant change in the expression of ERK1/2 in response to the inhibitors. The density of the bands was measured from several experiments, expressed as a ratio of phospho to total ERK1/2 levels and plotted in a histogram below the gel. *Brackets* indicate pairwise statistical comparisons of results from three independent experiments, yielding differences significant at the $**P < 0.001$ or the $*P < 0.05$ level; analysis was performed using Student's *t* test

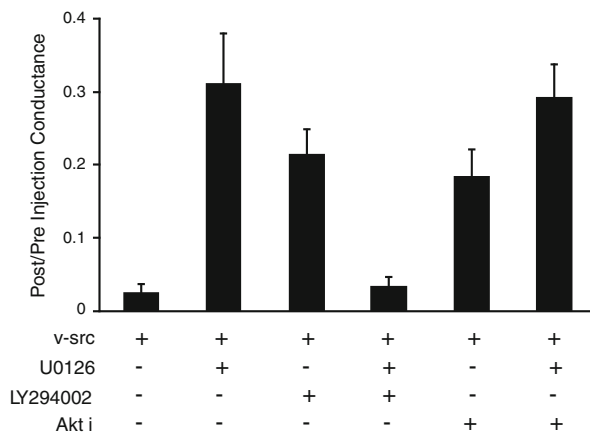


Fig. 7 The positive, but not the negative, regulatory effects of PI3K on src gating are mediated through Akt. As shown, inhibition of ERK by U0126 or PI3K by LY294002 each partially prevented v-src gating of Cx43. Inhibition of Akt by Akti had the same effect as PI3K inhibition, indicating that PI3K-induced closure of Cx43 is mediated through Akt. By contrast, the “rescue” of src gating by inhibition of both ERK and PI3K was not reproduced by a combination of ERK and Akt inhibition, indicating that the negative influence of PI3K on Cx43 gating by src is mediated through a pathway other than Akt

response to v-src has been consistently linked to direct phosphorylation of tyrosines in the C-terminal domain of Cx43, mapped to Y265, and a secondary site at Y247. Even after long-term expression of src, the phosphorylation of tyrosine in Cx43 remains in plaques (Solan and Lampe 2008); but it is not clear if the channels are gated or if other processes related to assembly are affected. Serine phosphorylation has also been associated with src expression but appears not to be required for long-term uncoupling (Lin et al. 2001). In contrast, the initial acute gating of Cx43 gap junction channels by src immediately after its expression has been linked to a “ball-and-chain” mechanism, triggered, not by tyrosine phosphorylation, but by ERK1/2 phosphorylation of serines in the C-terminal domain of Cx43 (Zhou et al. 1999). These specific sites were the same as those mapped by Warn-Cramer et al. (1996) in cellular responses to growth factors (i.e., serine S255, 279, 282), which suggests potential commonality in these gating mechanisms.

ERK Activation Alone Cannot Account for v-src Gating

In order to clearly dissect the temporal nature of src regulation of Cx43 and focus on the acute phase, which may be analogous to growth factor-induced gating of Cx43, we employed the *Xenopus* oocyte expression system. This allows for temporally controlled expression of the v-src kinase in cells that are already stably coupled by Cx43. Both mutation of ERK phosphorylation sites and inhibition of ERK activity (Zhou et al. 1999) (Fig. 1a) prevented v-src gating of Cx43, demonstrating the necessity of ERK for initial gating of Cx43 channels by v-src. However, we show here that this is not sufficient as activation of ERK by CA-MEK1 failed to close the Cx43 channels (Fig. 1c). This led us to investigate other pathways that could contribute to src gating. Our strategy was to combine the use of pharmacological blockers of different kinase pathways, which have been implicated in various studies of src action, with more specific knockouts of their target phosphorylation sites (in the case of ERK) or DN and constitutively active constructs (in the case of PKC). When these were not available, we used multiple inhibitors with different modes of action and specificities (in the case of PI3K). Using this double confirmation approach, we minimized the probability of off-target effects of the pharmacological agents alone (Davies et al. 2000; Anastassiadis et al. 2011).

Possible Roles for PKC in v-src Gating

Based on similar inhibitory effects of both BIM and an α -isotype-specific DN-PKC (Fig. 4a) on v-src gating of

Cx43, we concluded that closure of Cx43 channels by v-src also utilizes the PKC pathway. However, since deletion of all known PKC sites on Cx43 failed to affect gating (Fig. 2b), PKC must exert its effects on upstream events. This may be partially through interactions between PKC and ERK as we show that ERK phosphorylation is enhanced by CA-PKC and reduced by BIM (Fig. 3). However, all of PKC's effects cannot be mediated through ERK as CA-MEK1 induced significantly higher levels of ERK phosphorylation than either PKC isotype (Fig. 3) but with no effect on gating (Fig. 1a). In addition, both α and ϵ isoforms of PKC induced similar enhancement of ERK phosphorylation (data not shown), yet only the former affected gating (Fig. 4). The additive effect of U0126 and BIM (MEK and PKC inhibitors, respectively) on preventing v-src block of Cx43 (Fig. 2a) and the synergistic action of CA-MEK and CA-PKA α (Fig. 4b) suggest that MEK1/2 and PKC may affect closure of Cx43 channels through independent pathways. The specific PKC pathway remains to be elucidated, but it appears not to be mediated through direct phosphorylation of Cx43 on any of the identified consensus sites.

Complexity of PI3K Modulation of v-src Gating

PI3K, which is also known to be modulated by src activity (Penuel and Martin 1999), is involved in Cx43 gating, as inhibition of PI3K by two independent compounds attenuated v-src block of Cx43 (Fig. 5). This effect of PI3K appears to be mediated by Akt (Fig. 7), consistent with previous observations that have associated the PI3K-Akt pathway with both v-src and TNF α closure of Cx43 channels (Ito et al. 2010). In this previous case the effect was linked to Akt1. This is consistent with the effectiveness of Akti in preventing src gating, as this inhibitor targets Akts 1 and 2 (Barrett et al. 2005). Also, since inhibition of src gating by PKC was not further enhanced by inhibition of PI3K, these kinases may inhibit Cx43 through a common pathway. However, we also find that PI3K not only plays a role in promoting closure of the Cx43 channels by v-src, but also serves to antagonize this gating. This antagonistic effect appears not to involve Akt action, but to be mediated through ERK, as inhibition of PI3K caused an increase in ERK phosphorylation levels (Fig. 6) that may be sufficient to overcome the effects of MEK inhibition on src gating (Fig. 5).

An Integrated Model of v-src Gating of Cx43: A Delicate Balance

The same three pathways, Ras-Raf-ERK, PKC and PI3K, which we have identified as mediating initial v-src gating of Cx43, have frequently been implicated in many aspects

of growth factor signaling, with growing evidence of cross-talk between them at several levels. This is specifically true for the effects of growth factors on Cx43 coupling (Warn-Cramer et al. 1996; Hossain et al. 1999a, b). Consistent with these known pathways and the results presented here, one possible model for how v-src may regulate Cx43 coupling is presented in Fig. 8. Based on the demonstrated Ras-independent means of activation of ERK1/2 by PKC (Schonwasser et al. 1998; Kolch 2000), PI3K could activate MEK via PKC. This could explain why PI3K inhibitors alone partially blocked closure of Cx43 channels by v-src and why these inhibitors could not further enhance the effects of inhibition of PKC. Akt1 has also been implicated in the PI3K component of the src gating response of Cx43 (Ito et al. 2010), consistent with our finding that Akti can partially prevent acute src closure of Cx43 channels. The mechanism of Akt action on Cx43 has yet to be resolved, although Akt has been shown to phosphorylate Cx43 at both S369 and 373, leading to association of 14-3-3 with the Cx43 C-terminal domain (Park et al. 2007). This might be expected to affect aspects of Cx43 trafficking, but in the oocyte studies presented here the gating effects are acute and could suggest a more direct role of this interaction, or other effects of Akt, on the open state of Cx43 channels.

The mechanism by which the PI3K pathway also exerts antagonistic effects on src gating are less clear, although it appears this may be mediated through suppression of ERK activation based on the hyperphosphorylation of ERK that is observed in response to wortmannin. PI3K has been shown to inhibit the Ras-Raf-MEK1/2-ERK pathway via an antagonistic influence of Akt (Moelling et al. 2002), but this appears not to be the pathway in this study as Akti had no effect on reestablishing src gating in the presence of MEK inhibitors. These schizophrenic effects of PI3K, as both an activator and an inhibitor of the MEK1/2 pathway, are likely to be a critical aspect of the fine-tuning of ERK activity that appears to control Cx43 function in response to different mitogenic signals through convergence of several major signaling networks. This balance may be required to avoid activation of the negative feedback loop that has been shown to operate through the serine phosphorylation of SOS by ERK (Langlois et al. 1995). However, artificial elevation of ERK activity through CA-MEK expression, which produces maximal Cx43 phosphorylation, still does not result in gating. Hence, ERK-independent pathways must also be required for closure of Cx43 gap junction channels. The nature of these pathways remains to be elucidated (indicated by dotted arrows in Fig. 8), although we have eliminated some possibilities, such as direct PKC phosphorylation of Cx43 (Fig. 2a) and binding events, like ZO-1, at the very C-terminal tail of Cx43 (Fig. 4b).

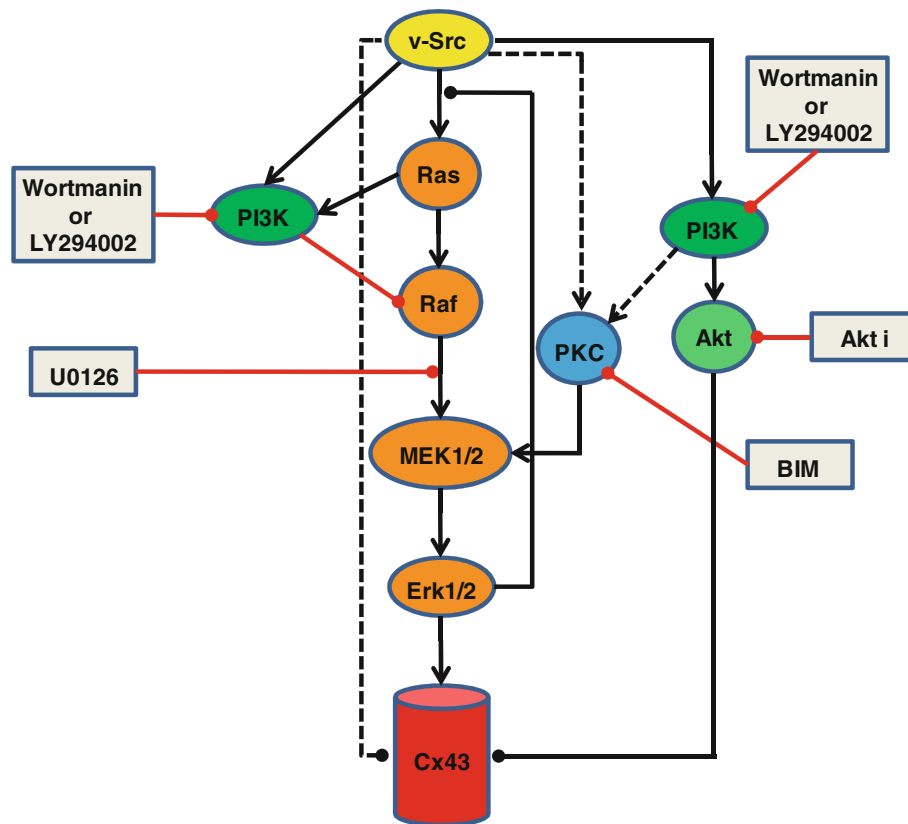


Fig. 8 Model for signaling pathways involved in v-src-induced initial closure of Cx43. The hypothetical model shown is based on the data presented here and established (*solid lines*) or proposed (*dashed lines*) connections from other studies (see text). We have noted only the simplest model consistent with the data and literature, and more complex connections are possible. Activating effects are indicated by *arrowheads* and inhibitory effects by lines with *solid circles*. The sites of action of the inhibitors (shown in *gray rectangles*) used in the current study are also indicated. The central player appears to be ERK1/2 (*orange pathway*). The PKC pathway (*blue*) can positively influence the activation of ERK1/2 but may also regulate Cx43 in a

manner dependent on its very C-terminal ZO1 binding domain. PI3K (*green*) can contribute to src gating through PKC (and ERK?) or through Akt (*light green*), possibly by direct effects on Cx43 that have been reported. PI3K can also antagonize src gating of Cx43 in a yet to be defined pathway that inhibits ERK activation. Together, these pathways may serve to regulate the levels of ERK1/2 activation, perhaps keeping it within a defined range so as not to activate the negative feedback loop of ERK to the activator of Ras. However, since full activation of ERK by CA-MEK fails to induce channel closure, pathways independent of ERK are likely to present parallel mechanisms for channel closure (*dashed lines*)

Comparison of Acute v-src vs. Growth Factor-Induced Closure of Cx43 Channels

The data presented here suggest that the initial action of v-src on Cx43 gap junction channels is similar to that of growth factors, with multiple control pathways regulating the process. This is typical of the checks and balances seen in other aspects of the mitogenic response. This initial uncoupling may serve to isolate cells from potential mitogenic inhibitory signals from neighbors or to allow accumulation of promitogenic activators within the cell. The effects of v-src diverge from those of growth factors in that the suppression of coupling becomes chronic. This effect, which has been linked to tyrosine phosphorylation of Cx43 in several systems, is likely mediated by a mechanism distinct from that of the initial gating response

to v-src studied here. Our analyses, including this distinction between acute and chronic gating, provide a basis for reconciliation of diverse reports in the literature. Chronic gating appears to be connected exclusively with tyrosine phosphorylation, while acute gating, which has been associated with Ras (Ito et al. 2006) and Cas (Shen et al. 2007) activity, appears to work through synergistic operation of the ERK, PKC and PI3K pathways.

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