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Modulation of *Xenopus* oocyte-expressed phospholemman-induced ion currents by co-expression of protein kinases

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Abstract

Phospholemman (PLM), the major sarcolemmal substrate for phosphorylation by cAMP-dependent kinase (PKA) protein kinase C (PKC) and NIMA kinase in muscle, induces hyperpolarization-activated anion currents in *Xenopus* oocytes, most probably by enhancing endogenous oocyte currents. PLM peptides from the cytoplasmic tail are phosphorylated by PKA at S68, by NIMA kinase at S63, and by PKC at both S63 and S68. We have confirmed the phosphorylation sites in the intact protein, and we have investigated the role of phosphorylation in the regulatory activity of PLM using oocyte expression experiments. We found: (1) the cytoplasmic domain is not essential for inducing currents in oocytes; (2) co-expression of PKA increased the amplitude of oocyte currents and the amount of PLM in the oocyte membrane largely, but not exclusively, through phosphorylation of S68; (3) co-expression of PKA had no effect on a PLM mutant in which all putative phosphorylation sites had been inactivated by serine to alanine mutation (SSST 62, 63, 68, 69 AAAA); (4) co-expression of PKC had no effect in this system; (5) co-expression of NIMA kinase increased current amplitude and membrane protein level, but did not require PLM phosphorylation. These findings point to a role for phosphorylation in the function of PLM.

Keywords: Ion channel regulation; Protein kinase; Phosphorylation

1. Introduction

Phosphorylation of sarcolemmal (SL) proteins is an important mechanism for regulation of cellular function. The cAMP-dependent protein kinase (PKA) phosphorylates several proteins in cardiac SL vesicles, the major one of which migrates with an apparent molecular weight of 15 000 Da on sodium dodecyl sulfate polyacrylamide gel electrophoresis. Protein kinase C (PKC), in contrast, phospho-

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rylates one substrate in great excess over others in cardiac SL vesicles, and this substrate also has an apparent molecular weight of 15 000 Da [1,2]. Phosphorylation by PKA and PKC is additive, suggesting that at least two different sites of the protein are phosphorylated. β-Adrenergic stimulation activates PKA in cardiac tissue [3], and α-adrenergic stimulation activates PKC [4]. In rodent hearts perfused with β-adrenergic [5] and α-adrenergic [6] agonists, the '15-kDa protein' is again the major SL substrate phosphorylated. ^{32}P-labeling of the protein, moreover, correlates with the positive inotropic response induced by either agonist. This '15-kDa protein' has been purified, the cDNA cloned, and named phospholemman (PLM) [7]. Multiple laboratories have demonstrated phosphorylation of PLM in cardiac membrane preparations and in intact cells [4,8–12].

As shown in Fig. 1, the amino acid sequence of PLM is highly conserved in dog, mouse, rat, and human [7,13]. In particular, the cytoplasmic domain and its four potential phosphorylation sites – S62, S63, S68, and S or T69 – are nearly completely conserved. In canine PLM, sites of phosphorylation by PKA and PKC have been identified. Using a synthetic peptide with the sequence of the cytoplasmic domain of PLM (amino acids 42–72), Lu et al. showed that PKA uniquely phosphorylates S68 [14]. Walaas and co-workers studied phosphorylation of synthetic PLM 58–72, and also found that S68 was phosphorylated by PKA and after adrenaline treatment [15]. Both groups found that PKC (or insulin treatment) phosphorylates S63 and S68. Importantly, prior phosphorylation by PKA may suppress phosphorylation of S63 by PKC [14]. This has implications regarding the role of PLM as an integrator of adrenergic inputs. PLM is also an excellent substrate for NIMA ('never in mitosis') kinase, which phosphorylates S63 [14]. This serine/threonine kinase, originally isolated from *Aspergillus nidulans*, is the product of the cell cycle regulatory gene *nimA* and is necessary for cells to enter mitosis [14,16].

The physiological role of PLM has not been defined. Reconstitution of PLM in lipid bilayers induces ion channels selective for the zwitterionic amino acid taurine [17]. Expression of PLM in *Xenopus* oocytes induces hyperpolarization-activated anion currents [18] although, since similar endogenous anion currents are present in oocytes, PLM may act in this system as a channel regulator [19]. The cytoplasmic domain of PLM (and therefore the phosphorylation sites) is not necessary for the formation of ion channels in lipid bilayers, but its presence does modulate channel function [20].

Although peptides derived from PLM are recognized substrates for protein kinase A, protein kinase C and NIMA kinase, the functional effects of PLM phosphorylation have not been investigated. In this study, we have examined the effects of PLM phosphorylation in the oocyte expression system. We find that co-expression of the three protein kinases tested have different effects on induced anion currents and the level of PLM expression, and that some, but not all, of these effects require intact PLM phosphorylation sites. We also demonstrate that the cytoplasmic domain is not necessary for induction of ion currents in *Xenopus* oocytes. Additionally, we have confirmed that the protein kinases phosphorylate full-length PLM at the same sites demonstrated in the peptide experiments.

2. Materials and methods

2.1. Analysis of phosphorylated residues

PLM protein was purified as previously described [21]. For phosphoamino acid and phosphopeptide analyses, PLM protein and peptide were phosphorylated in the presence of 50 μM [γ^{32}P]ATP and free radioactive ATP was separated on an AG1-X8 ion-exchange column in the presence of 30% acetic acid as described previously [22]. After repeated lyophilization in water, peptides were subjected to partial acid hydrolysis for phosphoamino acid analysis or repeated trypsin digestions for phosphopeptide analysis, followed by two-dimensional separation on thin layer chromatography as described previously [23].

2.2. Molecular reagents and immunoblotting

Wild-type, mutant and truncated PLM 1–43 cDNAs were prepared as previously described using canine PLM [20,24], and all new mutant PLM cDNAs were dideoxy sequenced. Protein kinase cDNAs encoded: (1) the catalytic subunit of PKA; (2) a constitutively active α-isozyme of PKC; or

(3) NIMA kinase. Immunoblotting and preparation of antibodies were performed as previously described [18,20,21].

2.3. Oocyte expression and electrophysiology

Our methods have been reported previously [18,19,24]. *Xenopus* oocytes were injected with RNA encoding wild-type or mutant PLM, and, in some, co-injected with RNA encoding PKA, PKC or NIMA kinase. After 24–96 h, currents were measured during hyperpolarizing steps from −10 mV (near the resting potential) using a two-microelectrode voltage clamp. Oocytes were then homogenized in a 1% Triton X-100 buffer for immunoblotting. Oocytes were manually defolliculated the day of the experiment. The bath solution contained (mM) NaCl (150), KCl (5), $CaCl_2$ (2), $MgCl_2$ (1.0), glucose (10), HEPES (10), pH 7.4 (NaOH); the flow rate was 3–4 ml/min. Experiments were performed at room temperature (20°C).

2.4. Statistical analysis

Current amplitudes were measured as the difference between initial and final levels of 2-s pulses to a test potential of −150 mV. In each batch of oocytes, we used two strategies to compare current amplitudes in oocytes expressing wild-type or mutant PLM alone to those in oocytes co-expressing protein kinases.

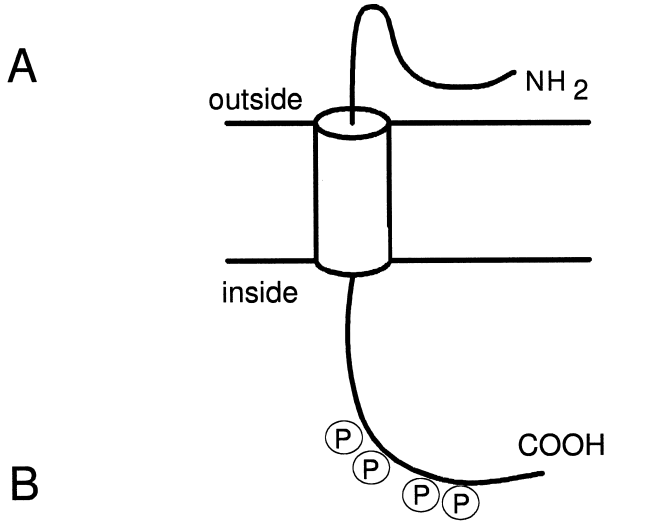
First, the mean current amplitude measured in oocytes expressing only wild-type or mutant PLM was determined. The current amplitude measured from each oocyte in that batch was normalized to this value, and box plots of the normalized current amplitudes were constructed. By definition, the mean normalized current in oocytes expressing wild-type or mutant PLM is 1, and the median varied. Pooled data sets usually failed a Kolmogorov–Smirnov test for normality and we used the non-parametric Mann–Whitney rank sum test to compare groups.

Second, the *t*-statistic for comparing two samples of unequal size and variance was calculated using current amplitudes from oocytes in the same batch, one set expressing wild-type or mutant PLM alone and the other co-expressing protein kinases. t was plotted as a function of the mean amplitude of normalized currents in oocytes expressing wild-type or mutant PLM alone. The value of t can be used to estimate the probability P of the null hypothesis, i.e. that co-expression of kinases had no effect on current amplitude. When $P < 0.05$, we concluded that kinase co-expression had a significant effect. The value of t for which $P < 0.05$ depends on the number of degrees of freedom (df), which is given by n_1+n_2-2. For infinite df, $t = \pm 1.96$. For our usual experiments with 15–25 df, t ($P < 0.05$) $\cong 2.1$, and these are the dotted lines in Figs. 5,8 and 10. Since the data were usually normally distributed within each group of oocytes (63 of 89 batches), points above (or below) the upper (or lower) line indicate individual experiments in which the effect of co-expressing protein kinase was to increase (or decrease) current amplitude significantly.

3. Results

3.1. Identification of PKA, PKC and NIMA phosphorylation sites on intact PLM

Phospholemman (PLM) has been previously shown to be phosphorylated by both cyclic AMP-dependent protein kinase (PKA) and protein kinase C (PKC) [7]. We have previously shown that these two kinases as well as the cell cycle regulated protein kinase NIMA phosphorylate a PLM peptide, PLM(42–72), which corresponds to the cytoplasmic domain of PLM [14]. To examine whether these kinases phosphorylate whole length PLM protein and to map their phosphorylation sites, PLM and the peptide PLM(42–72) were incubated with the kinases, followed by phosphoamino acid analysis and phosphopeptide analysis. Phosphoamino acid analysis indicated that all three kinases exclusively phosphorylated serine residue(s) both in whole protein and PLM(42–72) (Fig. 2A). There are three serines in the cytoplasmic domain of PLM at positions 62, 63 and 68. To pinpoint the phosphorylation site, phosphopeptide analysis was undertaken. Phosphopeptide analysis indicated that PKA and NIMA phosphorylated a single tryptic peptide, peptide 1 and peptide 2, respectively. These peptides were same no matter whether PLM or PLM(42–72) was used, as indicated by phosphopeptide mixing experi-



	N-terminus 1 10	Transmembrane domain 20 30
Canine PLM	EAPQEHDPFTYDYQSLR	IGGLIIAGILFILGILIVLS
Mouse PLM	EAPQEPDPFTYDYHTLR	IGGLTIAGILFILGILIILS
Human PLM	ESPKEHDPFTYDYQSLQ	IGGLVIAGILFILGILIVLS
Rat PLM	EAPQEPDPFTYDYHTLR	IGGLTIAGILFILGILIILS
RIC	...NENNPFYYDDTTLRKR	IGLVIAVGILTILIILTS
Rat CHIF	NGPVDKGSPFYYDWESLQ	LGGMIFGGLLCIAGIAMALS
Human MAT-8	NDLEDKNSPFYYDWHSLQ	VGGLICAGVLCAMGIIIVMS
gamma-subunit	AVQGTED.PFYYDYETVR	NGGLIFAALAFIVGLVIILS

	Cytoplasmic domain 40 50 60 70
Canine PLM	RRCRCKFNQQQRTGEPDEEEGTFRSSIRRLSTRRR
Mouse PLM	KRCRCKFNQQQRTGEPDEEEGTFRSSIRRLSSRRR
Human PLM	RRCRCKFNQQQRTGEPDEEEGTFRSSIRRLSTRRR
Rat PLM	KRCRCKFNQQQRTGEPDEEEGTFRSSIRRLSTRRR
RIC	GKCRQLSQFCLNRHR
Rat CHIF	GRCKCRRNHTPSSLPEKVTPLITPGSAST
Human MAT-8	AKCKCKFGQKSGHHPGETPPLITPGSAQS
gamma-subunit	KRFRCGAKRQHRQIPEDGL

Fig. 1. Topology and sequences of PLM and its family members. (A) Cartoon of the proposed topology based on the amino acid sequence. The amino-terminus is not accessible to proteases and is pictured apposed to the membrane. The cytoplasmic domain is accessible to proteases and houses the four indicated phosphorylation sites S62, S63 (substrate for PKC and NIMA), S68 (substrate for PKC and PKA) and S or T69. (B) Aligned amino acid sequences of PLM from four species, and for the four PLM family members RIC, CHIF, Mat-8, and the γ-subunit of the Na,K-ATPase. Note the extreme conservation of the cytoplasmic domain in PLM of all the species, which has little in common with the cytoplasmic domains of the family members other than the initiating (K/R) (K/R) C (K/R) C (K/R).

ments (Fig. 2B). However, as shown previously [14], the mobility of these two peptides was different when they were mixed and run on the same TLC plate, indicating that they belong to different peptides. Since NIMA exclusively phosphorylated Ser-63, PKA predominantly phosphorylated Ser-68, which contains the perfect consensus phosphorylation sequence RRXS for PKA. The identical two tryptic peptides were phosphorylated in PLM and PLM(42–72) by PKC (Fig. 2B). Phosphopeptide mixing experiments demonstrated that these two peptides co-migrated with peptides 1 and 2 which were independently phosphorylated by NIMA and PKA, respectively. As PKC still phosphorylated a peptide in which Ser-62 and Ser-68 had been substituted with alanines, peptide 2, obtained following phosphorylation by PKC, probably contained a phosphorylated Ser-63. These results indicated that on PLM protein, NIMA exclusively phosphorylates Ser-63, PKA phosphorylates Ser-68 and PKC phosphorylates both of these residues.

3.2. *Expression of PLM 1–43 induces hyperpolarization-activated Cl currents in Xenopus oocytes*

The cytoplasmic domain, which contains the phosphorylation sites, modulates PLM channel activity in lipid bilayers [20]. Therefore we investigated whether the cytoplasmic domain was necessary for PLM expression to induce hyperpolarization-activated currents in *Xenopus* oocytes. Fig. 3 shows families of whole oocyte currents obtained using two-microelectrode voltage clamping. Each is the average of records from four to six oocytes from the same frog. Panel A shows small endogenous currents in uninjected oocytes; panel B shows currents induced by expression of PLM 1–43; and panel C shows currents induced by expression of intact PLM. PLM 1–43 expression induces currents similar to those induced by intact PLM – they are hyperpolarization-activated with slow activation, and there is no current decay during these two second pulses.

Panel D, a current–voltage relationship representing data from three frogs, shows that the threshold and voltage dependence of activation is very similar for the expressed intact and PLM 1–43. Panel E shows averaged currents at a test potential of −150 mV. The currents have been normalized such that the peak currents are the same. There was a subtle, but consistent, difference in the activation kinetics. As we previously described, currents induced by intact PLM (dashed line) activate after a delay. This delay

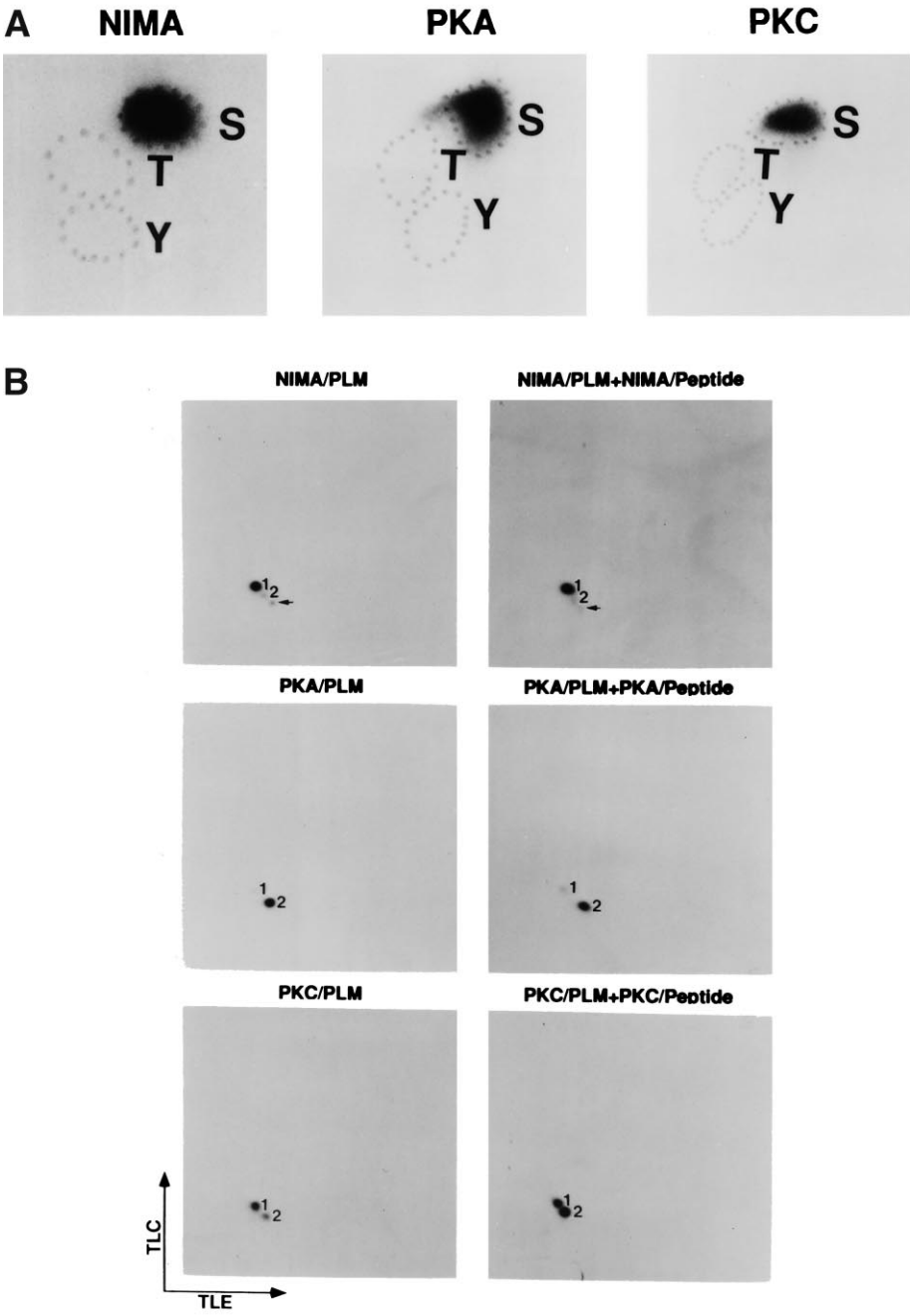


Fig. 2. Determination of phosphorylation sites of PLM by three protein kinases. (A) Phosphoamino acid analysis. PLM was phosphorylated by the different protein kinases as indicated and phosphorylated peptides were isolated and subjected to acid hydrolysis, followed by two-dimensional separation on thin-layer chromatography plates. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. (B) Phosphopeptide analysis. PLM and peptide PLM(42–72) were phosphorylated by indicated kinases and the phosphorylated protein and peptide were isolated, followed by subjecting to repeatedly digestion with trypsin and two-dimensional separation on thin-layer chromatography plates separately (left panel) or combinatively (right panel).

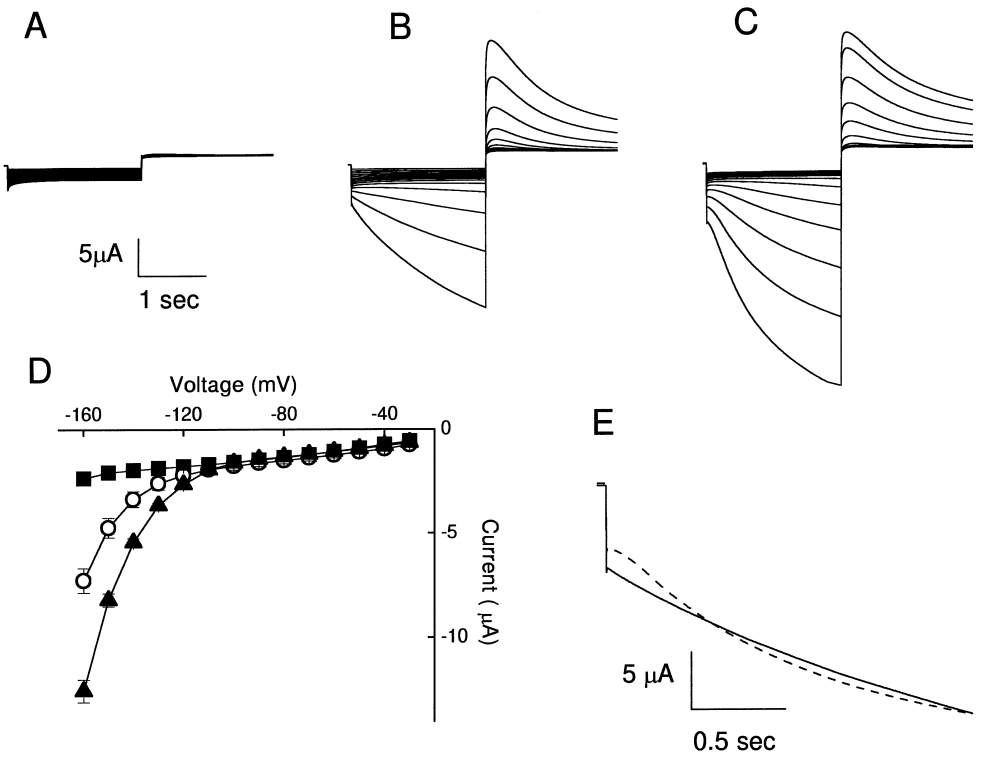


Fig. 3. Effects of expression of PLM 1–43 on hyperpolarization-activated currents in *Xenopus* oocytes. (A–C) Families of currents from uninjected oocytes (A), oocytes expressing PLM 1–43 (B), and oocytes expressing wild-type PLM (C). Each panel shows mean currents from four to six oocytes from the same frog. Two-second hyperpolarizing clamp pulses to test potentials between −40 and −160 mV were applied in 10-mV decrements from a holding potential of −10 mV before repolarization to 40 mV. (D) Current–voltage relationships for uninjected oocytes (filled squares) and oocytes expressing wild-type PLM (filled triangles) and PLM 1–43 (open circles). Currents were measured at the end of the hyperpolarizing pulses, and the data points are means (±S.D.) from 12–15 oocytes from three frogs. (E) Currents at a test potential of −150 mV recorded from oocytes expressing wild-type PLM (dashed line) and PLM 1–43 (solid line). Each record was obtained from a single oocyte from the same frog, and the amplitudes have been normalized to emphasize differences in kinetics.

is not apparent with currents induced by PLM 1–43 (solid line). The currents induced by PLM 1–43 could be fit adequately to a single exponential decay (τ 2.3 s), whereas the currents induced by wild-type PLM have a sigmoidal delay and are better described by:

$$I(t) = Io + A(1-\exp(-t/\tau_1))^N \exp -t/\tau_2$$

where, in the example, τ_1 is 0.5 s and τ_2 is 5.5 s, and N is 1.7. We have reported a similar effect of point mutations in the transmembrane domain of PLM [24]. We found no other consistent changes in current kinetics, though there was variability in the current waveforms after expression of wild-type or mutant PLM and co-expression of kinases. For example, although the currents in Fig. 4A and B appear to activate with different kinetics, the times to half-activation are very similar – 0.75 versus 0.74 s (A) and 0.62 versus 0.58 s (B).

These experiments established PLM 1–43, which cannot be phosphorylated, as a useful reagent. Kinases that modulate currents induced by oocyte expression of PLM 1–43 cannot act by phosphorylation of the cytoplasmic domain PLM molecule.

3.3. Co-expression of PKA increases the amplitude of $I_{Cl(PLM)}$

Fig. 4 shows the effect of co-expressing PLM and the catalytic subunit of PKA on the amplitude of hyperpolarization-activated Cl currents in *Xenopus* oocytes ($I_{Cl(PLM)}$). Expression of PKA alone did not induce hyperpolarization-activated oocyte currents (not shown). The data were derived from the

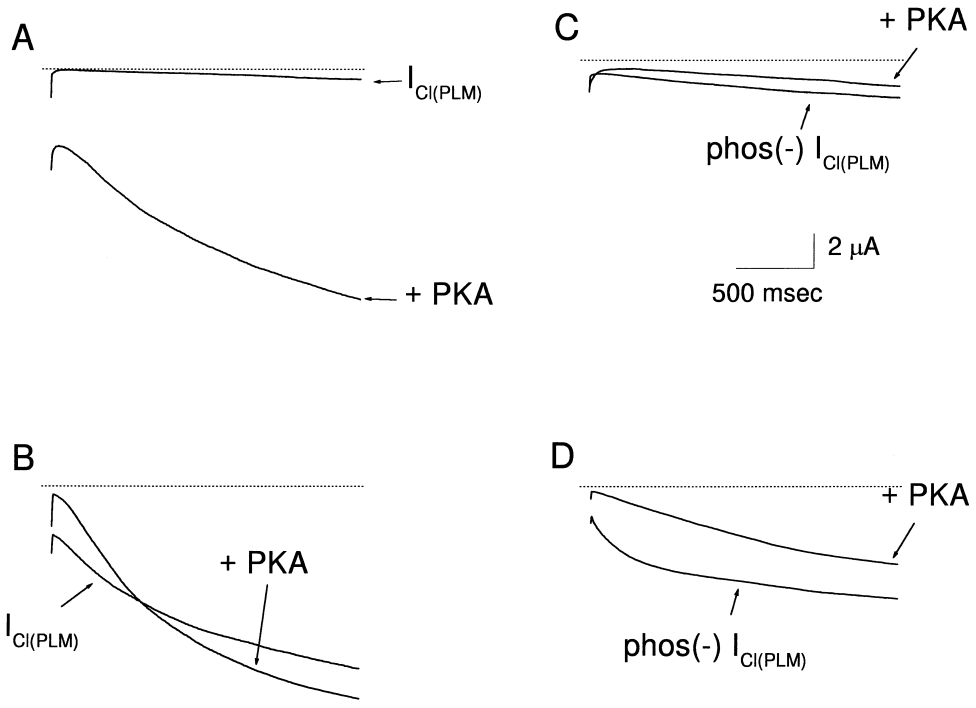


Fig. 4. Effects of PLM and PKA expression on hyperpolarization-activated currents in *Xenopus* oocytes. Each panel shows superimposed currents at a test potential of −150 mV from an oocyte expressing PLM ($I_{Cl(PLM)}$), or phos(−) PLM (phos(−) $I_{Cl(PLM)}$) alone, and from an oocyte co-expressing PKA (+PKA). A and C show data from oocytes studied early after injection, when $I_{Cl(PLM)}$ was <1.5 μA, and B and D were recorded later when $I_{Cl(PLM)}$ was >1.5 μA. The dotted lines represent 0 μA. Co-expression of PKA enhanced $I_{Cl(PLM)}$ when the amplitude of $I_{Cl(PLM)}$ in oocytes expressing PLM alone was small (A), but had no further effect when $I_{Cl(PLM)}$ was large (B). Co-expression of PKA with phos(−) PLM had little effect when currents induced by phos(−) PLM were small (C), and sometimes induced a reduction in current when this current was large (D).

same batch of oocytes, injected with identical quantities of PLM mRNA and, where applicable, PKA mRNA. Early (1–2 days) after mRNA injection when $I_{Cl(PLM)}$ was small, PKA co-expression resulted in increased current amplitude (A). In oocytes studied later (3–5 days after injection) when $I_{Cl(PLM)}$ was large, co-expression of PKA led to no further increase (B).

3.4. The effect requires the presence of PLM phosphorylation sites

To test the hypothesis that the PKA effect was caused by PLM phosphorylation, we co-expressed PKA with a PLM mutant in which the four major phosphorylation sites were disabled by mutagenesis. Fig. 1B shows the amino acid sequence of the cytoplasmic tail of PLM, which contains the four sites, S62, S63, S68 and T69. We constructed a phosphorylation-deficient (phos(−)) PLM mutant (SSST 62/63/68/69 AAAA) which disables all four sites. As shown in Fig. 4, expression of this phos(−) mutant PLM successfully induced currents in oocytes (C,D). Co-expression of PKA did not result in increased current amplitude in oocytes expressing phos(−) mutant PLM either early (C) or late (D) after mRNA injection. In fact, the current amplitude was slightly reduced.

Fig. 5A shows a box plot summary of normalized current amplitudes for 19–66 oocytes from 6–15 frogs. The data were dichotomized according to mean baseline $I_{Cl(PLM)}$ amplitude in oocytes expressing PLM mRNA alone as ≤ 1.5 μA, or >1.5 μA. Oocytes with baseline $I_{Cl(PLM)} \leq 1.5$ μA were those studied early after injection and those with baseline $I_{Cl(PLM)} > 1.5$ μA were studied later, 3–5 days after injection. In oocytes studied early, mean $I_{Cl(PLM)}$ was 0.5 ± 0.1 μA and co-expression of PKA resulted in a 10- to 30-fold increase in $I_{Cl(PLM)}$ ($P < 0.001$, first and second boxes). By contrast, in oocytes studied

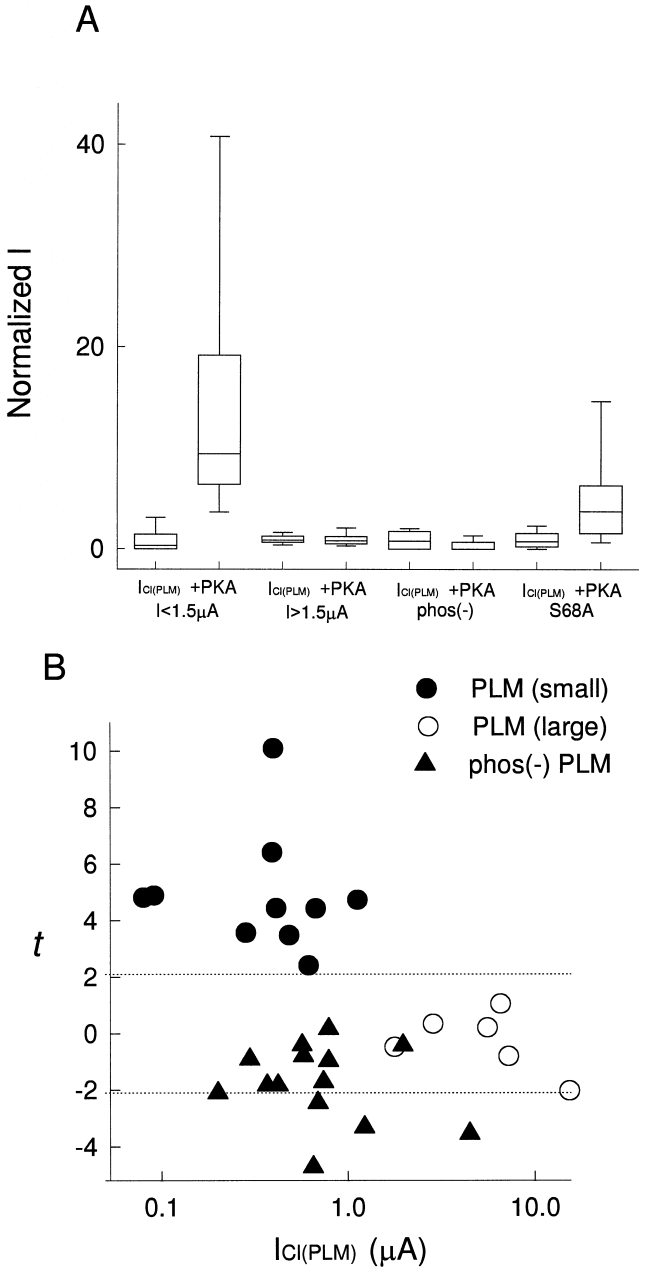


Fig. 5. (A) Effects of PLM and PKA expression on normalized hyperpolarization-activated current amplitudes in *Xenopus* oocytes. In the box plot symbols, the horizontal line is the median, 50% of data points lie within the box and 90% lie within the hatches. First and second symbols: amplitude of $I_{Cl(PLM)}$ was increased by PKA co-expression soon after PLM RNA injection when $I_{Cl(PLM)}$ was $\leq$1.5 μA ($n=66$ and 69, 10 frogs; medians 0.3 and 9.4; $P<0.001$). Third and fourth symbols: amplitude of $I_{Cl(PLM)}$ was unchanged by PKA co-expression later after RNA injection when $I_{Cl(PLM)}$ was >1.5 μA ($n=34$ and 40, six frogs; medians 0.9 and 0.8; $P=0.6$). Fifth and sixth symbols: amplitude of $I_{Cl(PLM)}$ induced by expression of phos(−) PLM was reduced by co-expression of PKA ($n=71$ and 61, 15 frogs; medians 0.8 and 0; $P<0.001$). Seventh and eighth symbols: inactivation of the PKA phosphorylation site (S68) by mutation to alanine (S68A) diminished the effect of PKA co-expression on $I_{Cl(PLM)}$ ($n=30$ and 19, three frogs; median current amplitudes 0.8 and 3.7; $P<0.001$ for the increase in S68A $I_{Cl(PLM)}$ amplitude induced by PKA; $P<0.001$ for the comparison of S68A $I_{Cl(PLM)}$ plus PKA with wild-type $I_{Cl(PLM)}$ plus PKA). (B) Effect of PKA on the amplitude of induced hyperpolarization-activated currents in individual batches of oocytes. t, a measure of the effect of PKA co-expression on current amplitude, is plotted as a function of mean current amplitude in oocytes expressing PLM alone. Each data point represents a single batch of oocytes and the dotted lines are $t=\pm2.1$, which approximates $P<0.05$. Co-expression of PKA with wild-type PLM resulted in a significant increase in current amplitude in 10 of 10 batches when the mean amplitude in oocytes expressing PLM alone was $\leq$1.5 μA (filled circles). When $I_{Cl(PLM)}$ was >1.5 μA (open circles), PKA co-expression did not result in a significant difference in any batch of oocytes. In oocytes expressing phos(−) PLM (filled triangles) there was a significant reduction in amplitude in four of 15 batches.
←

later, mean $I_{Cl(PLM)}$ was 6.4 ± 0.8 μA, and co-expression of PKA had no significant effect (third and fourth boxes). There was no significant difference in the peak PKA stimulated current in oocytes with small or large baseline $I_{Cl(PLM)}$ (5.3 ± 0.5 μA compared with 5.9 ± 1.0 μA). In oocytes co-expressing PKA and phos(−) mutant PLM, the large increase in current amplitude seen with wild-type PLM was abolished and overall current amplitude was reduced ($P<0.001$, fifth and sixth boxes).

Fig. 5B shows the magnitude of the effect of PKA co-expression in individual batches of oocytes. The t statistic, used as a measure of the difference in current amplitude between oocytes expressing PLM (or phos(−) PLM) alone and those co-expressing PKA, is plotted as a function of the mean current amplitude in oocytes expressing PLM (or phos(−) PLM) alone. Each data point represents a separate batch of oocytes. Points lying above or below the dotted lines represent a batch of oocytes where co-expression of PKA resulted in a significant increase or decrease in current amplitude. For small $I_{Cl(PLM)}$ (≤1.5 μA, filled circles), PKA co-expression resulted in a significant increase in current amplitude in 10 of 10 batches, whereas for large baseline $I_{Cl(PLM)}$

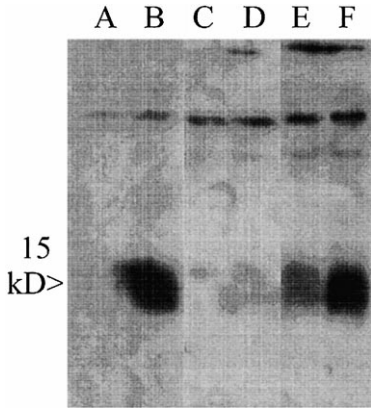


Fig. 6. Immunoblot of oocyte membranes probed with poly anti-PLM antibodies. (A) Membranes from oocytes injected with PLM mRNA alone. (B) Oocytes coinjected with PKA mRNA. (C) Phos(−) PLM mRNA alone. (D) Phos(−) PLM mRNA and PKA mRNA. (E) S68A PLM mRNA alone. (F) S68APLM mRNA and PKA mRNA.

(>1.5 μA, open circles) there was never a significant effect of PKA co-expression. In oocytes expressing phos(−) PLM (filled triangles), PKA co-expression never resulted in an increase in current amplitude. There was a significant reduction in current amplitude in 4 of 15 batches of oocytes; in the other batches, there was no significant effect. These data suggest that phosphorylation of the cytoplasmic domain of PLM by PKA leads to increased current amplitude, since when the phosphorylation sites were disabled, the effect of PKA to increase amplitude was lost.

3.5. Elimination of S68, the PKA phosphorylation site, reduces but does not prevent the functional effect of PKA co-expression in Xenopus oocytes

In vitro, PKA phosphorylates the PLM carboxy-terminal peptide at S68 (this report and [14,15]). To test the idea that the increase in $I_{Cl(PLM)}$ induced by co-expression of PKA is mediated via phosphorylation at this site, we co-expressed PKA with the PLM mutant S68A. The PKA effect should be blocked in this mutant if in vitro results translate to intact pro-

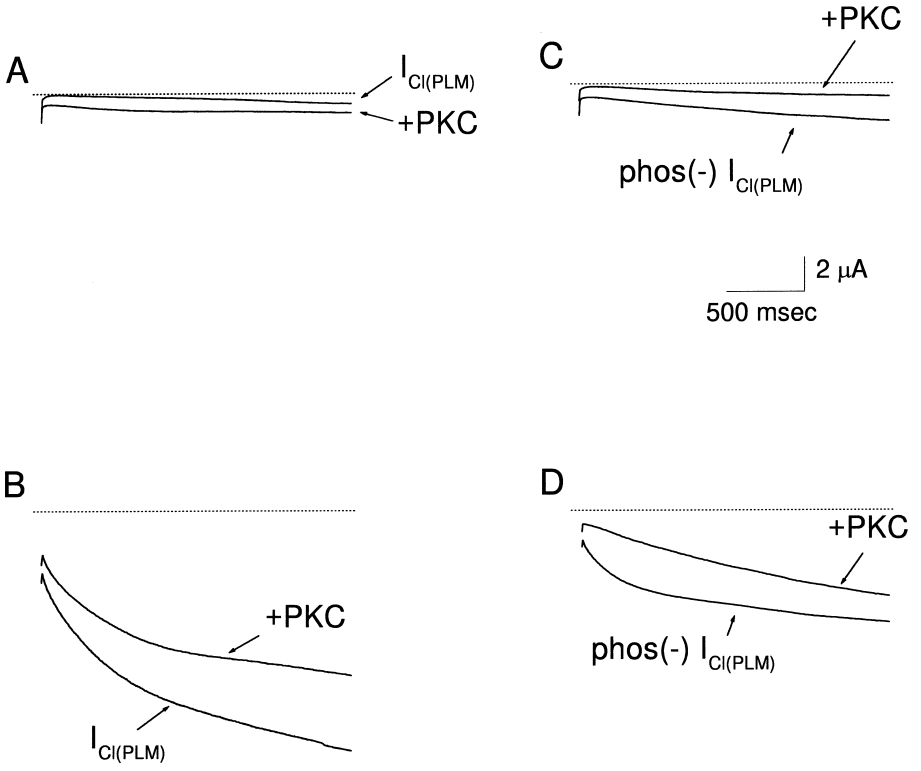


Fig. 7. Effects of PLM and PKC expression on hyperpolarization-activated currents in *Xenopus* oocytes. Co-expression of wild-type PLM and PKC had little effect on current amplitude when $I_{Cl(PLM)}$ in oocytes expressing PLM alone was small (A), but when $I_{Cl(PLM)}$ was larger, PKC co-expression reduced current amplitude (B). A similar effect was observed in oocytes co-expressing phos(−) PLM and PKC (C,D).

tein expressed in cells. Surprisingly, co-expression of PKA with PLM S68A increased $I_{Cl(PLM)}$ amplitude ($P < 0.001$ compared with oocytes expressing S68A PLM alone, Fig. 5A, seventh and eighth boxes). The increase, however, was significantly less than the effect on currents induced by wild-type PLM. PKA co-expression led to only a 5-fold increase in median amplitude of currents induced by S68A PLM compared to a 28-fold increase in median amplitude of currents induced by wild-type PLM ($P < 0.001$ for the difference). This suggests that the effect of PKA to enhance $I_{Cl(PLM)}$ is mediated partially, but not solely through phosphorylation of S68.

3.6. Co-expression of PKA increases the level of membrane PLM

Fig. 6 is an immunoblot probed with affinity-purified polyclonal antibodies to PLM 58–72, the 15 carboxyl-terminal amino acids of PLM. Each lane contains membranes from five oocytes previously used for measurement of current amplitude. The first pair of lanes shows the results for oocytes expressing wild-type PLM alone (lane A) or co-expressing PKA (lane B). The currents recorded from the oocytes in lane A were very small (Fig. 4A) and PLM expression was correspondingly very small. There was a large increase in the amount of immunoreactive PLM when PKA was co-expressed, however, which corresponds to the large increase in current (Fig. 4A). The second pair of lanes shows results for oocytes expressing phos(−) PLM. The currents recorded from the oocytes were small (Fig. 4C) and expression was correspondingly small (lane C). Co-expression of PKA (lane D) had no effect of PLM expression. The third pair of lanes shows results for oocytes expressing S68A PLM. Baseline S68A PLM expression was larger than wild-type PLM or phos(−) PLM (compare lanes A, C and E). PKA co-expression increased the level of immunoreactive PLM, but not to the same degree as in wild-type PLM (compare lanes B and F). The results were confirmed in three other immunoblots for each condition, and were the same using affinity-purified antibodies to PLM 1–15, the 15 amino-terminal amino acids, and using a monoclonal antibody with an epitope in the transmembrane and cytoplasmic domain. These data indicate that the effect of PKA to in-

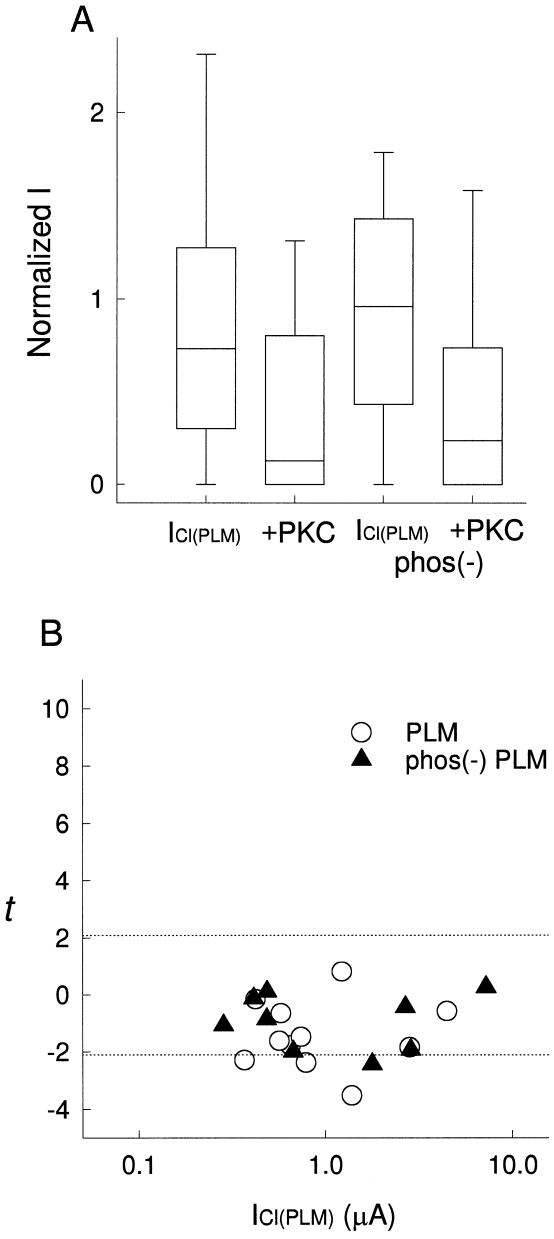


Fig. 8. (A) Effects of PLM and PKC expression on normalized hyperpolarization-activated Cl current amplitudes in *Xenopus* oocytes. First two symbols: amplitude of $I_{Cl(PLM)}$ was reduced by co-expression of PKC ($n = 71$ and 56 oocytes from the same 11 frogs; medians of normalized current amplitudes 0.7 and 0.1; $P = 0.001$). Third and fourth symbols: amplitude of $I_{Cl(PLM)}$ induced by expression of phos(−) PLM was similarly reduced ($n = 66$ and 70 oocytes from the same 9 frogs; medians of normalized current amplitudes were 1.0 and 0.2; $P < 0.001$). (B) The effect of PKC co-expression to reduce induced currents was significant in three of 11 batches expressing wild-type PLM and in only one of nine batches expressing phos(−) PLM.

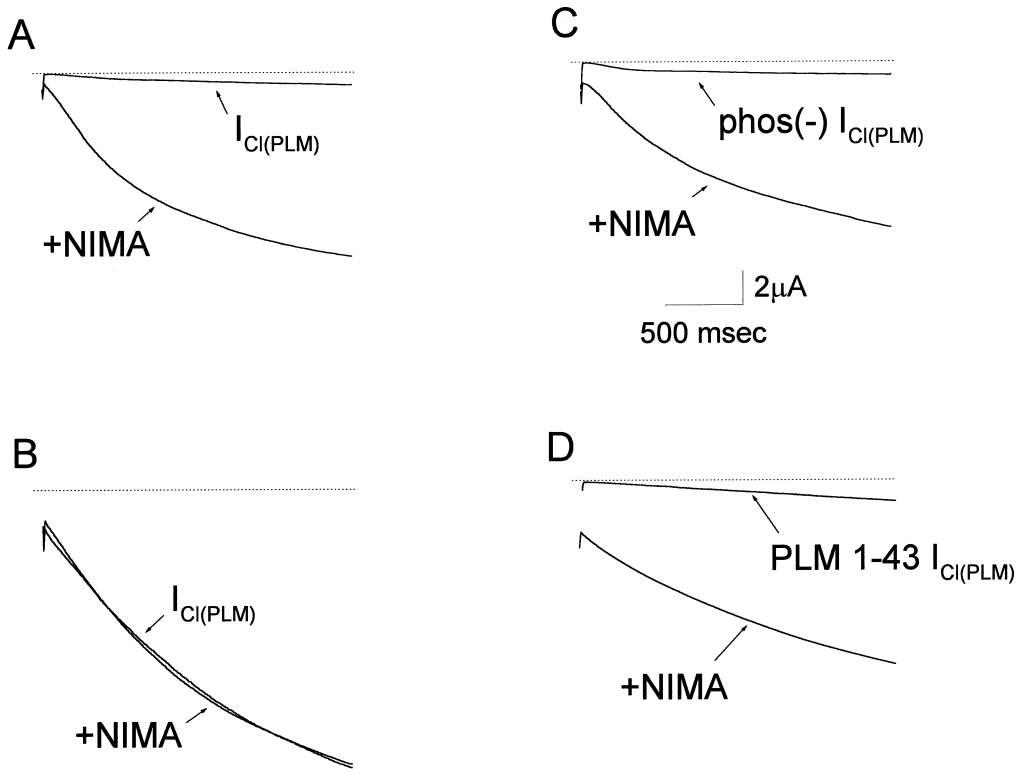


Fig. 9. Effects of PLM and NIMA expression on hyperpolarization-activated currents in *Xenopus* oocytes. Co-expression of NIMA induced an increase of $I_{Cl(PLM)}$ in oocytes expressing wild-type (A) and phos(−) PLM (B) when the current in oocytes expressing PLM (or phos(−) PLM) alone were small. When $I_{Cl(PLM)}$ was larger (B), the effect was less pronounced. Co-expression of PLM 1–43 with NIMA resulted in increased $I_{Cl(PLM)}$ (D).

crease $I_{Cl(PLM)}$ is accompanied by increased PLM expression in the oocyte membrane. In phos(−) oocytes where PKA co-expression did not lead to an increase in $I_{Cl(PLM)}$, there was no increase in expression.

3.7. Co-expression of PKC reduces the amplitude of $I_{Cl(PLM)}$

Expression of PKC alone did not induce oocyte currents (not shown). Fig. 7 shows that co-expression of PKC with both wild-type and phos(−) PLM did not increase, but instead resulted in a small decrease in the amplitude of $I_{Cl(PLM)}$. Fig. 8A shows summary results for 56–71 oocytes from 9–11 frogs. The effect of co-expressing PKC was a significant reduction of current amplitude in oocytes expressing either wild-type or phos(−) mutant channels ($P < 0.001$ for the pooled results in each case), though the reduction was significant in only three of eleven batches of oocytes expressing wild-type PLM and in one of nine batches expressing phos(−) PLM (Fig. 8B). Thus, in contrast to co-expression of PKA, PKC had no functional effect on $I_{Cl(PLM)}$ attributable to PLM phosphorylation in this expression system. Rather, the small reduction in $I_{Cl(PLM)}$ we observed was independent of the presence of the recognized PLM phosphorylation sites.

3.8. Co-expression of NIMA kinase increases the amplitude of $I_{Cl(PLM)}$

Expression of NIMA kinase alone did not induce oocyte currents (not shown). Fig. 9A and B show the effect of co-expression of NIMA kinase on $I_{Cl(PLM)}$. Current amplitude increased when $I_{Cl(PLM)}$ in oocytes expressing PLM alone was small (A), but the effect was much less marked when $I_{Cl(PLM)}$ was large (B). Surprisingly, co-expression of NIMA with phos(−) PLM, where all potential phosphorylation sites had been disabled, also resulted in a marked increase in current amplitude (C). To confirm this result, we

tested the ability of NIMA co-expression to increase the amplitude of currents induced by PLM 1–43, in which the PLM cytoplasmic domain phosphorylation sites are deleted. Again, NIMA co-expression increased the amplitude of induced currents (D).

Fig. 10A shows summary results for 36–96 oocytes isolated from 5–9 frogs. When $I_{Cl(PLM)}$ was small, co-expression of NIMA resulted in a 4–5-fold increase in current amplitude ($P < 0.001$, first two boxes) whereas no such effect was observed when baseline current was large. Co-expression of NIMA with phos(−) PLM resulted in a more than two-fold increase in current amplitude ($P < 0.001$, third and fourth boxes), and co-expression of NIMA with PLM 1–43 resulted in an increase of $I_{Cl(PLM)}$ of similar magnitude ($P < 0.001$, fifth and sixth boxes).

Fig. 10B shows that the NIMA effect was largest when induced currents were small. This was true for oocytes expressing wild-type PLM (filled circles, ≤1.5 μA; open circles, >1.5 μA), phos(−) PLM (filled triangles) and PLM 1–43 (open squares).

4. Discussion

To examine the role of phosphorylation of the cytoplasmic domain of PLM, we studied hyperpolarization-activated ion currents induced by PLM expression in *Xenopus* oocytes. We induced PLM phosphorylation by co-expressing PKA, PKC and NIMA kinase, each of which has been previously shown to phosphorylate peptides derived from the cytoplasmic

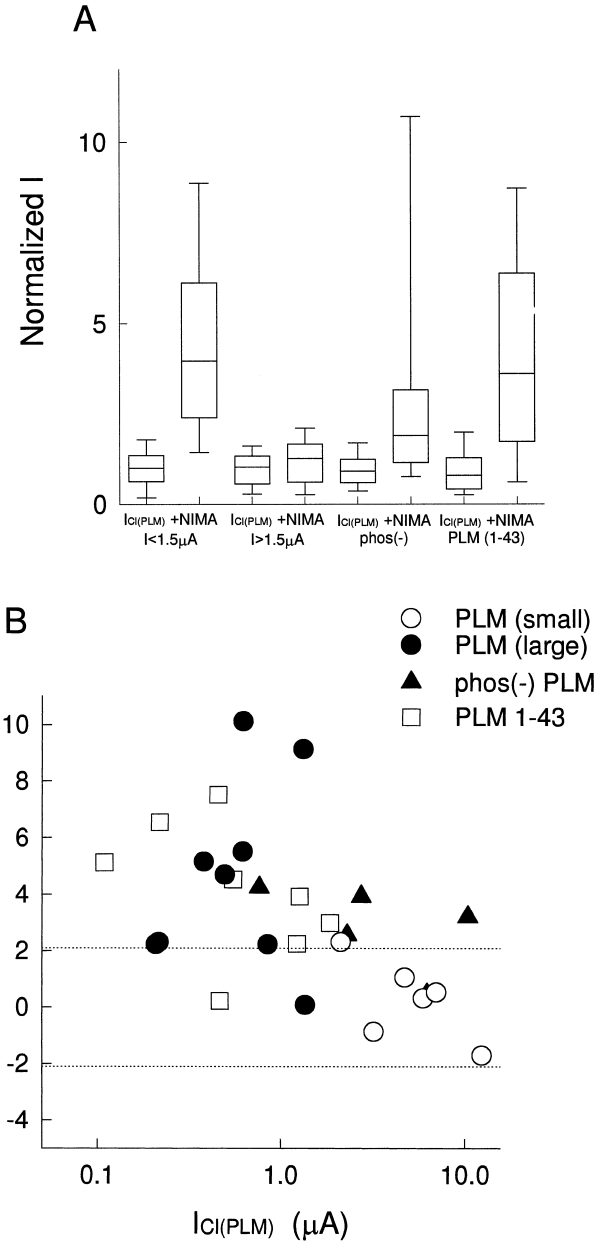


Fig. 10. (A) Effects of PLM and NIMA expression on hyperpolarization-activated Cl currents in *Xenopus* oocytes. First two symbols: amplitude of $I_{Cl(PLM)}$ was increased by expression of NIMA kinase when $I_{Cl(PLM)}$ was ≤1.5 μA ($n = 62$ and 65 oocytes from the same nine frogs; medians of normalized current amplitudes were 1.0 and 4.0; $P < 0.001$). Third and fourth symbols: co-expression of NIMA had no significant effect on $I_{Cl(PLM)}$ amplitude when $I > 1.5$ μA ($n = 36$ and 38 oocytes from the same six frogs; median current amplitudes were 1.0 and 1.3; $P = 0.2$). Fifth and sixth symbols: co-expression of NIMA increased the amplitude of $I_{Cl(PLM)}$ in oocytes expressing phos(−) PLM ($n = 39$ and 30 from the same five frogs; median current amplitudes were 0.9 and 1.9; $P < 0.001$). Seventh and eighth symbols: co-expression of NIMA increased the amplitude of $I_{Cl(PLM)}$ in oocytes expressing PLM 1–43 ($n = 89$ and 96 from the same eight frogs; median current amplitudes 0.8 and 3.6; $P < 0.001$). (B) Effect of NIMA co-expression on the amplitude of induced hyperpolarization activated currents in individual batches of oocytes. In oocytes expressing wild-type PLM, NIMA co-expression induced an increase in $I_{Cl(PLM)}$ in eight of nine batches when $I_{Cl(PLM)}$ was ≤1.5 μA (filled circles), but was without effect in six of six batches when $I > 1.5$ μA (open circles). In oocytes expressing phos(−) PLM, NIMA co-expression enhanced current amplitude in four of five batches (filled triangles). In oocytes expressing PLM 1–43, NIMA co-expression enhanced current amplitude in seven of eight batches. Overall, the effect of NIMA was most marked when $I_{Cl(PLM)}$ was smallest.

domain of PLM. Our most important findings are:

1. PKA, PKC and NIMA kinases phosphorylate the same sites in intact PLM as they do in PLM peptides;
2. co-expression of PKA increased current amplitude and the amount of PLM present in the oocyte membrane;
3. the effect of PKA to enhance $I_{Cl(PLM)}$ required the presence of PLM phosphorylation sites especially (but not exclusively) the major PKA phosphorylation site S68;
4. co-expression of PKC reduced current amplitude, but this effect did not require the PLM phosphorylation sites;
5. co-expression of NIMA kinase increased current amplitude and this effect too did not require the presence of PLM phosphorylation sites.

We have previously demonstrated that PLM expression induces Cl currents in *Xenopus* oocytes [18]. This finding can be interpreted in more than one way: PLM might be an ion channel, or might be a regulator of endogenous oocyte Cl channels [19]. Point mutations in the PLM molecule alter the kinetics [18] and the voltage-sensitivity [19] of the current, suggesting that PLM itself constituted the channel molecule. Subsequent descriptions, however, of endogenous currents with characteristics very similar to those induced by PLM expression, support the regulator hypothesis [19,25–29]. The presence of these endogenous channels renders the *Xenopus* oocyte an inappropriate expression system for testing the hypothesis that PLM is itself a channel. We have demonstrated that highly purified recombinant PLM forms anion-selective channels in planar lipid bilayers [17,20,21], but the precise physiological significance of this in the oocyte expression system is unclear. While our working hypothesis is that PLM is a regulator of endogenous oocyte channels, all of the data can also be explained if PLM itself forms ion channels in oocyte membranes, or PLM is a channel protomer that forms heteromultimeric channels with an as yet unidentified oocyte homolog [19].

Phosphorylation is prominent in PLM regulation. The cytoplasmic carboxyl-terminus in which the phosphorylation sites are located (Fig. 1) is readily accessible to protease [20], suggesting it may be freely mobile in the cytoplasm. One potential effect of phosphorylation is reducing the net charge of the cytoplasmic domain, which houses many positively charged residues. This might result in an alteration in the conformation or mobility of the cytoplasmic domain leading to changes in PLM function. Its presence is not essential, however, as expression of PLM 1–43 induced hyperpolarization-activated currents in *Xenopus* oocytes (this report), and purified recombinant PLM 1–43 formed channels in bilayers [20]. The present work supports the idea that phosphorylation of the cytoplasmic domain plays an important modulatory role in PLM function as an ion channel or regulator.

In *Xenopus* oocytes, where PLM is hypothesized to act as a regulator of endogenous anion channels, phosphorylation of the cytoplasmic domain after co-expression of kinases results in changes in the amplitude of the induced currents. The effects of the three kinases we tested (PKA, PKC, and NIMA kinase) were different. PKA co-expression increased current amplitudes when baseline $I_{Cl(PLM)}$ was small, an effect which required the presence of the phosphorylation sites in the cytoplasmic domain. The increased current amplitude induced by co-expression of PKA correlates with increases in the amount of PLM in the oocyte membrane, as demonstrated in the immunoblot. This finding is not likely to be due to a change in the affinity of the antibody for phosphorylated PLM, as it was evident using three antibodies whose epitopes span the length of the PLM molecule. How phosphorylation increases the level of membrane PLM is not demonstrated in these studies. One possible explanation is that phosphorylation results in translocation of PLM from the cytosol to the membrane. Since phosphorylation would make the molecule less hydrophobic, it is likely that other membrane proteins participate in the process, in keeping with the view that PLM acts in concert with other molecules in inducing currents in oocytes. Alternatively, phosphorylation could result in an increase in total cellular PLM. Our experiments do not distinguish these possibilities, as measurement of cytoplasmic PLM in the complex oocyte cytoplasmic milieu was not possible.

The increase in $I_{Cl(PLM)}$ and PLM expression in-

duced by co-expression of PKA was absent in a PLM mutant lacking all four cytoplasmic domain phosphorylation sites, and significantly reduced in a PLM mutant lacking the PKA phosphorylation site identified in vitro (S68A). The absence of an increase in $I_{Cl(PLM)}$ or PLM expression in oocytes expressing phos(−) channels indicates that phosphorylation of the cytoplasmic domain is necessary for correct expression of the functional effect of PKA on wild-type channels. The reduced effect of PKA co-expression in oocytes expressing S68A implicates S68 in the mechanism of the PKA effect. Other mediators of the PKA effect are not demonstrated in these studies, but are likely to include the other cytoplasmic phosphorylation sites, since inactivation of all four sites abolished the PKA effect. Other mechanisms could be involved, however. For example, the intact peptide may assume a conformation not available to the short synthetic peptides used previously, allowing another potential phosphorylation site to be active. Another possible explanation is that PKA activates intermediary kinases that phosphorylate PLM at other sites. The effects on $I_{Cl(PLM)}$ of co-expression of PKA were not simply the effect on the endogenous oocyte anion current of co-expression of any heterologous protein, as has been demonstrated in oocytes for some proteins [28], since the effects we observed could be abolished by inactivation of the PLM phosphorylation sites.

The reduction of $I_{Cl(PLM)}$ induced by PKC co-expression was not dependent on intact PLM phosphorylation sites, indicating that this action is likely to be independent of PLM phosphorylation at these sites. PKC certainly phosphorylates PLM peptides, but this effect does not increase PLM expression in this system, so the consequence of PLM overexpression – up-regulation of the endogenous oocyte current – is not apparent. NIMA kinase co-expression had the same effect as PKA with the surprising difference that the amplitudes of currents induced by a mutant phos(−) PLM lacking the four phosphorylation sites or by PLM 1–43 which lacks nearly all of the cytoplasmic domain were increased to the same extent as currents induced by wild-type PLM. This suggests that phosphorylation of a related protein by NIMA kinase is sufficient to increase membrane levels of non-phosphorylated PLM, and underscores the complexity of studying the regulation and function of PLM expressed in *Xenopus* oocytes.

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