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Co-localization of the α-subunit of BK-channels and c-PLA$_2$ in GH3 cells

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Abstract

Large conductance, calcium-activated potassium channels (maxi K- or BK-channels) can be regulated by arachidonic acid produced by c-Phospholipase A_2 (c-PLA$_2$). Since in every excised patch from GH3 cells where there was BK-channel activity, treatment with either a stimulator or inhibitor of c-PLA$_2$ resulted in a corresponding increase or decrease in BK-channel activity, we hypothesized that there must be a close association between BK-channel proteins and c-PLA$_2$ in the cell membrane. To test this hypothesis, we first determined whether the two proteins would co-immunoprecipitate. We then used confocal imaging of fluorescently tagged proteins to determine where in the cells BK-channel proteins and c-PLA$_2$ co-localize. The α-subunit of the BK-channel was strongly co-immunoprecipitated by c-PLA$_2$ antibodies, suggesting that most of the BK channel α-subunits are associated with c-PLA$_2$. This interaction was not affected by pharmacologically inhibiting c-PLA$_2$ suggesting that the association does not require functionally active c-PLA$_2$. Following dual immunohistochemical labeling and confocal microscopy, image analysis revealed that in the cytosol there was some co-localization, but most of the c-PLA$_2$ was separate from BK-channel proteins. On the other hand, the c-PLA$_2$ and BK-channel proteins at the plasma membrane were strongly co-localized. Immunoprecipitation experiments conducted with plasma membrane proteins support these findings. We conclude that c-PLA$_2$ is likely physically associated with BK-channel proteins at the cell surface.

Keywords: BK-channels; Phospholipase A_2; Confocal imaging; Immunoprecipitation; Immunohistochemistry; Arachidonic acid

One important type of CNS ion channel is the large conductance Ca^{2+}-activated potassium (BK) channel with at least six distinct functional types of BK-channels within the CNS [1]. BK-channels in the CNS are essential to maintain normal rhythmic activity of repetitively firing neuronal cells and they are involved in regulating the level of secretion from neurosecretory cells [2,3]. BK-channels in the neurosecretory GH3 cell line have been well characterized by us and others [4–10]. BK-channels are the large conductance (150–400 pS) member of the Ca^{2+}-activated potassium channel family, which also includes small conductance (SK; 8–20 pS) and intermediate conductance (IK; 50–100 pS) channels. For many years, it was thought that the very sensitive Ca^{2+} activation of all of these channels was due to binding of multiple Ca^{2+} ions directly to the ion channel protein, itself [11,12]. However, several years ago a major fraction of SK channel calcium sensitivity was found to depend upon Ca^{2+}-binding to calmodulin and calmodulin activation of the channel rather than binding of Ca^{2+} to the channel protein [13]. At about the same time, we reported that a similar situation appeared true for BK-channels: although [Ca_i^{2+}] does bind to and impart some basal activity to BK-channels in GH3 cells, the steep P_0 vs. [Ca_i^{2+}] relationship which is characteristic for these channels in native cells involves Ca^{2+}-binding to and activation of c-PLA$_2$ [14]. Because of the physiological importance of BK-channels, we are particularly interested in the cellular signaling mechanisms responsible for controlling the activity of BK-channels. In previous work, we have demonstrated that arachidonic

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acid produced by cytosolic phospholipase A_2 is actually responsible for the observed increases in BK-channel open probability in response to increases in [Ca_i^{2+}] GH3 cells [14].

Until recently it has been thought that optimal activation of c-PLA_2 requires both increases in intracellular Ca^{2+} and phosphorylation [15,16]. It was also thought that c-PLA_2 resided in the cytosol and translocated to the cell membrane only in response to large increases in intracellular Ca^{2+} [15,17]. However, in many cells, including GH3 cells, there appears to be some c-PLA_2 constitutively associated with the membrane which is active and capable of producing arachidonic acid [18]. In previous work using single channel methods to examine BK-channels [18], we found that in every excised patch from GH3 cells where we observed BK-channel activity, treatment with either a stimulator (mellitin) or inhibitor (aristolochic acid or AACOCF3) of c-PLA_2 resulted in a corresponding increase or decrease in BK-channel activity, we hypothesized that there is a close association between BK-channel proteins and c-PLA_2 in the cell membrane. To test this hypothesis we conducted experiments to determine whether the two proteins would co-immunoprecipitate and confocal imaging experiments to determine whether co-localization could be demonstrated visually. We also used confocal imaging to determine what effects resulted from increases in intracellular Ca_i^{2+} which is known to stimulate translocation of c-PLA_2 from the cytosol to the plasma membrane [17,19]. Finally, we examined the association in the presence of c-PLA_2 inhibitors to determine whether the association required fully functional c-PLA_2.

Methods

Cell culture. The GH3 cell line was obtained from American type culture collection (ATCC; Rockville, Maryland) and was grown at 37 °C in a 5% CO_2 atmosphere in Dulbecco's minimum essential media (DMEM) supplemented with 15% heat-activated horse serum, 2.5% fetal bovine serum, and 2 mM glutamine. Cells from passages between 25 and 45 were used in the experiments described in this manuscript. Cells for immunoprecipitation experiments were grown to confluence in T-75 culture flasks. Cells for immunohistochemistry studies were plated in 24-well plates containing 12 mm round glass coverslips, which had been pretreated with poly lysine.

Antibodies and special reagents. Primary antibodies to α-BK-channels (host: rabbit) and c-PLA_2 (host: mouse) were obtained from Chemicon International, Temecula, CA. A BK-channel antibody designed specifically for immunoprecipitation was a generous gift from Dr. Irwin Levitan, (U PA). Horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences, Piscataway, NJ. Alexafluor® Fluorescent goat-anti rabbit (AlexaFluor-488 to label BK-channels) and goat-anti mouse (AlexaFluor-568 to label c-PLA_2) antibodies as well as TO-PRO-3® nuclear stain and ProLong® antifade reagent were obtained from Molecular Probes, Eugene, OR. A/G Agarose beads were obtained from Santa Cruz Biotechnology, Inc. Santa Cruz, CA. Biotinylation reagents (EZ-Link NHS–SS–biotin) were obtained from Pierce Biotechnology, Inc. Rockford, IL. The two c-PLA_2 inhibitors, aristolochic acid and AACOCF3, were obtained from Biomol Research Laboratories (Plymouth Meeting, PA).

Whole cell lysate immunoprecipitation procedure. Immunoprecipitation experiments were conducted based on the methods previously described for the precipitation of α and β BK-channel subunits [20]. One flask containing approximately 10^7 cells was lysed in a gentle lysis buffer (150 mM NaCl, 50 mM Hepes, 1 mM EGTA, 1 mM EDTA, 100 mM sodium fluoride, 10 mM tetrasodium pyrophosphate, 100 μM sodium orthovanadate, 10% glycerol, 1% TX-100, pH 7.4, with 300 nM *p*-nitrophenyl phosphate, 1 mM PMSF, 1 mM Pefablock®, 10 mg/ml aprotinin, and 10 mg/ml leupeptin) for 30 min at 4 °C. A second flask of cells was lysed using RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1.0 mM EDTA, 0.1% SDS, 1.0% Triton X-100, and 1.0% sodium deoxycholate. The protease inhibitors described above were also added).

Following cell lysis (1 h at 4 °C), the suspension was centrifuged at 4000*g* for 10 min to precipitate unlysed cells. Thirty microliters of protein A/G PLUS-agarose beads were added to the supernatant from the cells lysed using the gentle lysis buffer and mixed at 4 °C for 2 h with gentle agitation to remove any non-specifically binding proteins and any non-solubilized membrane fragments. A/G-Agarose was pelleted by centrifugation at 4000*g* for 5 min at 4 °C. The supernatant was carefully removed and saved for immunoprecipitation. Such a procedure was unnecessary for cells lysed in RIPA buffer since the higher concentrations of detergent in RIPA were sufficient to solubilize all membrane components. For immunoprecipitations (IP), 1 ml cell lysate, and 10 μl c-PLA_2 antibody (conc: 1.6 mg/ml) were incubated overnight at 4 °C. Thirty microliters of protein A/G PLUS-agarose was added and mixed at 4 °C for 2 h with gentle agitation. A/G-Agarose was pelleted by centrifugation at 4000*g* for 5 min at 4 °C. The supernatant was carefully removed and saved for analysis by Western Blot. The pellet was washed three times with 30 μl Laemmli sample buffer (Tris, pH 6.8; 0.1% SDS, 10% glycerol, and 0.025% Bromphenol blue). The resuspended sample, which also contained 20 mM DTT, was heated at 95 °C for 5 min and the pellet fraction centrifuged 4000*g* for 5 min prior to gel loading and Western blot analysis.

Western blot analysis. Western blot analysis was completed as previously described [14]. Thirty microliters of the eluent and original supernatant was loaded in each lane on a 7.5% polyacrylamide gel. Following electrophoresis (approximately 1 h at 150 V), the proteins were transferred to nitrocellulose. The nitrocellulose blots were then probed with primary antibodies for α-BK-channels (1:1000). Blots were then washed and incubated with a secondary antibody (1:160,000). The secondary antibody used was goat anti-rabbit IgG, coupled to alkaline phosphatase (Kirkegaard and Perry Laboratories, MD), and the antigen antibody complex was detected by a chemiluminescent detection system CDP-star (Tropix, MA) and Kodak 2000M camera system (Eastman Kodak Company, Rochester, NY).

Membrane protein precipitation procedure. Surface proteins on approximately 2×10^7 GH3 cells were labeled with biotin as described previously [21]. After cell lysis, binding to Avidin beads, and separation from non-biotinylated proteins, the labeled surface proteins were released from the Avidin beads with 200 mM glycine buffer (pH 2.5). After neutralization, the resultant solution was subjected to the immunoprecipitation and Western protocols described above.

Immunohistochemistry procedure. GH3 cells were plated on 12 mm coverslips pre-coated with polylysine (Fisher scientific, Atlanta, GA) at a density of approximately 10^5. Media were removed and cells were washed with phosphate-buffered saline (PBS) containing Ca^{2+} and Mg^{2+} three times for 5 min each. All subsequent PBS washes also contained $Ca2^+$ and Mg^{2+}. For experiments with A23187, the cells were treated with 10 μM A23187 for 15 min at room temperature prior to fixation. Cells were fixed with a 5-min exposure to 4% paraformaldehyde at room temperature. This treatment was followed by three 5-min washes with PBS. The cells were then permeablized by treatment with 0.1% Triton X for 5 min. Following three 5-min PBS washes; the cells were blocked with 3% goat serum for 30 min. Cells were then exposed to the primary antibody for BK-channels (1:1000; host rabbit) and/or c-PLA_2 (1:1000; host mouse) in 3% goat serum for 1 h at room temperature. Following three, 5 min washes with PBS the appropriate fluorescent secondary antibodies (1:10,000) were introduced and incubated for 1 h at room temperature. Negative controls, designed to assess non-specific binding, were conducted in parallel. For these controls, cells were treated as described above except that they were not

exposed to primary antibody. Cells were then incubated for 3 min with TO-PRO-3® in PBS. The cells were then washed with PBS for 5 min each. Finally the coverslips were mounted on glass microscope slides using ProLong® antifade reagent to minimize photo-bleaching. The slides were stored in the dark at −20 °C until they could be examined using a Zeiss LSM 510 NLO META confocal microscope (Zeiss, Thornwood, NY). Preliminary experiments were conducted with dilutions of 1:50, 1:100, 1:250, 1:500, and 1:1000 with each of the primary antibodies, and 1:500, 1:1000, and 1:10,000 with each of the secondary antibodies in an effort to optimize the visualization of the two proteins of interest and minimize the degree of non-specific binding (background).

Assessment of co-localization of BK and c-PLA$_2$ fluorescent label. We used the "co-localization finder" plugin [22] for the image analysis program, "Image J" [23], to analyze our images for co-localization of the labels for BK antibodies and for c-PLA$_2$ antibodies. This plugin displays a correlation diagram as a scatterplot for two images (8 bits, same size). Drawing a rectangular selection on this diagram allows the user to identify pixels that contain both channel 1 (BK fluorescence—green) and channel 2 (c-PLA$_2$ fluorescence—red). These pixels will then be highlighted in white on a RGB merge of the original red and green images. We restricted the analysis to pixels having ratios of intensity values in the two channels greater than 0.1, but then set the threshold above the background levels which are relatively easy to identify in the scatterplot as the cluster of points near the origin. We calculated Pearson's correlation coefficient (R_r) according to Eq. (1) for the entire scatterplot.

$$R_r = \frac{\sum_i (S1_i - S1_{\text{aver}}) \cdot (S2_i - S2_{\text{aver}})}{\sqrt{\sum_i (S1_i - S1_{\text{aver}})^2 \cdot \sum_i (S2_i - S2_{\text{aver}})^2}}, \tag{1}$$

where $S1$ is the signal intensity of pixels in the first channel and $S2$ is the signal intensity of pixels in the second channel. The values $S1$ (average) and $S2$ (average) are average values of pixels in the first and second channels, respectively. In Pearson's correlation, the average pixel intensity values are subtracted from the original intensity values. As a result, the value of this coefficient ranges from −1 to 1, with a value of −1 representing a total lack of overlap between pixels from the images, and a value of 1 indicating perfect overlap. Pearson's correlation coefficient accounts only for the similarity of shapes between the two images; therefore, we also calculated the Overlap coefficient (R) according to Eq. (2). This value ranges between 0 and 1, and is not sensitive to intensity variations in the image analysis.

$$R = \frac{\sum_i S1_i \cdot S2_i}{\sqrt{\sum_i (S1_i)^2 \cdot \sum_i (S2_i)^2}}. \tag{2}$$

The product of channel intensities in the numerator returns a significant value only when both values belong to a pixel involved in co-localization (if both intensities are greater than zero). As a result, the numerator in Eq. (2) is proportional to the number of co-localizing pixels. In a similar manner, the denominator of the Overlap equation is proportional to the number of pixels from both components in the image, regardless of whether or not they are co-localized. The Overlap coefficient is relative insensitive to differences in signal intensities between various components of an image. The disadvantage of using the Overlap coefficient is the influence of the ratio between the number of image features in each channel. To alleviate this dependency, we also calculated the Overlap sub-coefficients, termed k_1 and k_2, in order to express the degree of co-localization as two separate parameters:

$$R^2 = k_1 \cdot k_2, \qquad k_1 = \frac{\sum_i S1_i \cdot S2_i}{\sum_i (S1_i)^2}, \qquad k_2 = \frac{\sum_i S1_i \cdot S2_i}{\sum_i (S2_i)^2}. \tag{3}$$

The overlap coefficients, k_1 and k_2, describe the differences in intensities between the channels for overlapping pixels, with k_1 being sensitive to the differences in the intensity of channel 2 (green signal), while k_2 depends linearly on the intensity of the pixels from channel 1 (red signal).

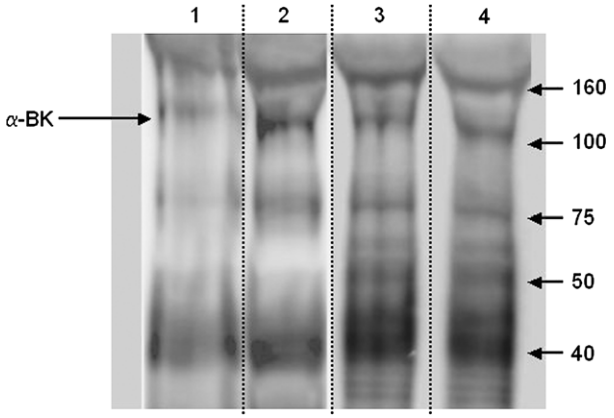

Fig. 1. α-Subunits of the BK-channel associate with c-PLA2. Following cell lysis with either RIPA buffer (lane 1) or a gentle lysis buffer (lane 2), we immunoprecipitated c-PLA$_2$ using a commercially available antibody, solubilized the precipitate, and resolved the immunoprecipitated proteins on an SDS–gel. We then used a commercially available anti-BK-channel antibody to detect the BK-channel α subunit as a 105 KDa band. The α subunit of the BK-channel was strongly co-immunoprecipitated with the c-PLA$_2$, suggesting that most BK-channel α subunits are associated with c-PLA$_2$. After lysing the cells with gentle lysis buffer and prior to the immunoprecipitation, the lysate was pre-absorbed with protein A–agarose beads to remove non-specifically binding proteins and any non-solubilized membrane fragments. Lanes 3 and 4 are identical to lane 2 except that the cells for lane 3 were pre-treated with AACOCF3 (15 μM) for 15 min prior to harvest and the cells for lane 4 were pre-treated with aristolochic acid (250 μM) for 15 min prior to harvest. As described for lane 2, α subunit of the BK-channel was strongly co-immunoprecipitated with the c-PLA$_2$, suggesting that the association does not require fully functional c-PLA$_2$.

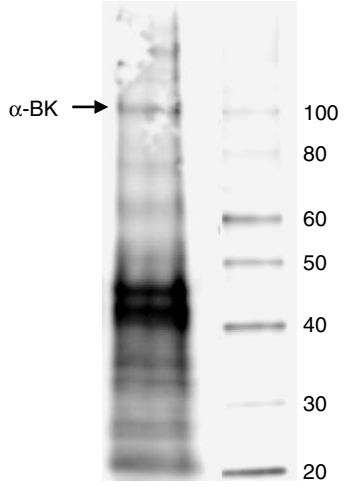

Fig. 2. α-Subunits of the BK-channel associate with c-PLA2 at the plasma membrane. After labeling the membrane proteins from GH3 cells with biotin and precipitating with avidin-coated beads, we released the proteins and then immunoprecipitated with an antibody to c-PLA$_2$, solubilized the precipitate, and resolved the proteins on an SDS–gel. We used a commercially available anti-BK-channel antibody which could detect BK-channel α-subunit in Western blots as a 105 KDa band. The α-subunit of the BK-channel was co-immunoprecipitated with the c-PLA$_2$, suggesting that BK-channel α-subunits are associated with c-PLA$_2$ at the plasma membrane. This is a representative blot from a series of three experiments that produced the same results.

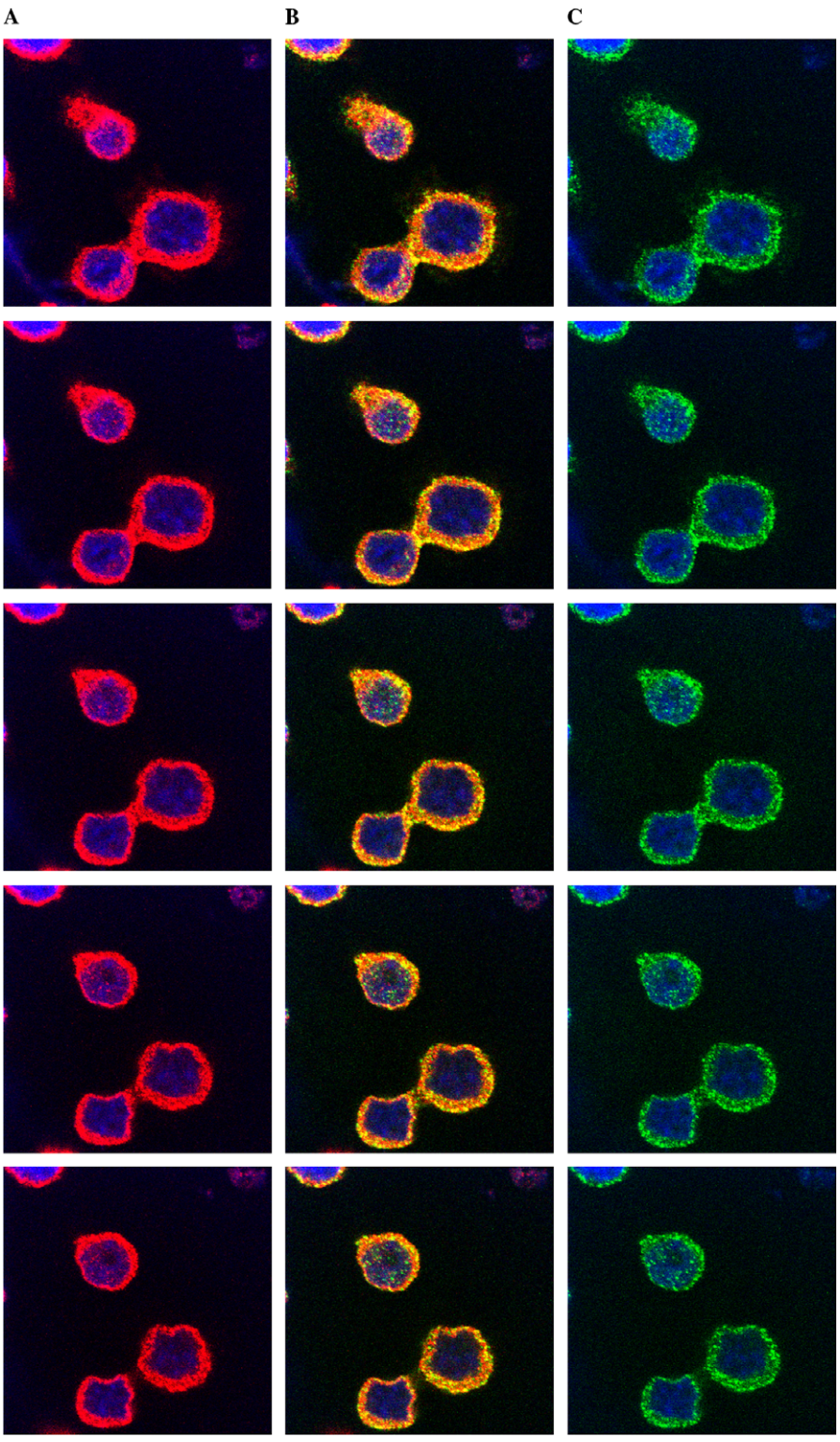

Fig. 3. c-PLA$_2$ is in close association with BK-channels in GH3 cells. GH3 cells were stained with primary antibodies directed toward c-PLA$_2$ (host: mouse) and the α-subunit of BK-channels (host: rabbit). Following treatment with TO-PRO-3® (blue nuclear stain) and fluorescent secondary antibodies c-PLA$_2$ (red) and BK-channel (green) the cells were examined using confocal microscopy using a Zeiss LSM 510 NLO META confocal microscope. The five images in (A–C) are sequential 0.25 μ slices near the middle of the cells. (A,C) Reveal the presence of c-PLA$_2$ and BK-channel proteins, respectively, while (B) is the composite image. The yellow pixels in the composite image demonstrate the close association of the two proteins. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Results

Co-immunoprecipitation of c-PLA$_2$ and BK-channels in GH3 cells

As we have previously reported, any manipulation which prevents activation or expression of PLA$_2$ makes BK-channels markedly insensitive to intracellular calcium (although they still require nanomolar concentrations to maintain any activity) [14]. If this observation is correct, then c-PLA$_2$ and BK-channel proteins at the cell surface must be either in extremely close proximity or actually physically associated. We first tested this hypothesis using immunoprecipitation followed by Western blotting. Because we were unsure whether the association between α-BK and c-PLA$_2$ was strong enough to withstand lysis with RIPA buffer which contains substantial concentrations of detergent, we conducted parallel experiments lysing cells with a gentle lysis buffer which contains low levels of detergent (but which requires a pre-absorption procedure to eliminate non-specific binding of protein and non-solubilized membrane components—see Methods). We immunoprecipitated c-PLA$_2$ (see Fig. 1) using a commercially available antibody, solubilized the precipitate in RIPA buffer, and resolved the immunoprecipitated proteins on an SDS–gel. We then used a commercially available anti-BK-channel antibody to detect the BK-channel α subunit as a 105 KDa band. The α subunit of the BK-channel was strongly co-immunoprecipitated with the c-PLA$_2$, suggesting that most BK-channel α subunits are associated with c-PLA$_2$. The first two lanes in Fig. 1 illustrate that BK α is immunoprecipitated in both gentle lysis buffer (containing only non-ionic detergents) and RIPA buffer (which contains low levels of stronger ionic detergents). As might be expected, the band from the cells treated with RIPA buffer is weaker in intensity, nonetheless, the

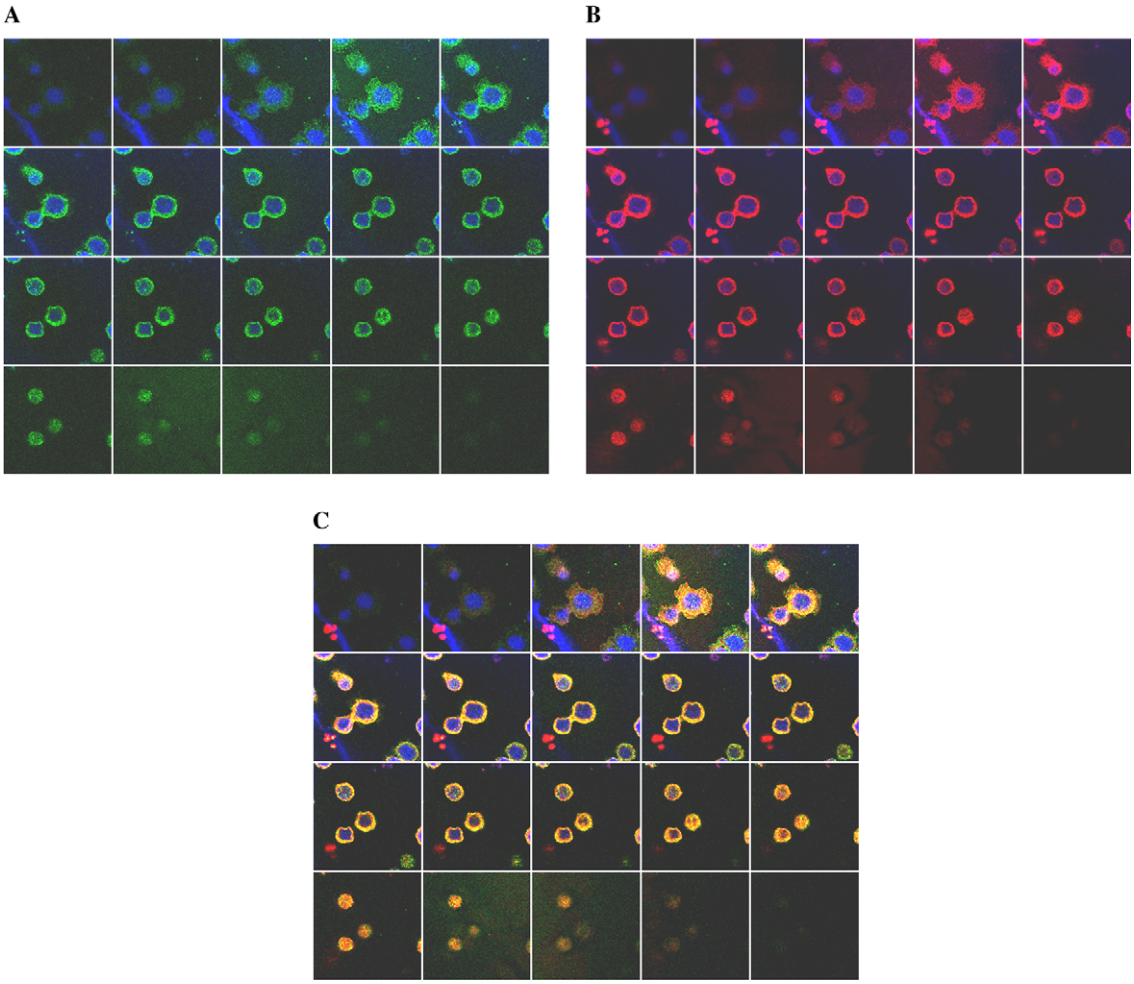

Fig. 4. c-PLA$_2$ and BK-channels are ubiquitously distributed throughout the cell but closely associated when both are at the plasma membrane in GH3 cells. As described in Fig. 3, GH3 cells were stained with primary antibodies directed toward c-PLA$_2$ (host: mouse) and the α-subunit of BK-channels (host: rabbit). Following treatment with TO-PRO-3® (blue nuclear stain) and fluorescent secondary antibodies c-PLA$_2$ (red) and BK-channel (green) the cells were examined using confocal microscopy using a Zeiss LSM 510 NLO META confocal microscope. The twenty images in (A–C) are sequential 0.25 μ slices from the bottom to the top of GH3 cells. (A,B) reveal the presence, BK and c-PLA$_2$ channel proteins, respectively, while (C) is the composite image. The yellow pixels in the composite image demonstrate the close association of the two proteins (a result supported by the co-immunoprecipitation in Figs. 1 and 2). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

fact that it is detectable implies that the interaction between BK α and c-PLA_2 must be strong enough to withstand the detergents present in RIPA buffer. To examine the hypothesis that the association between c-PLA_2 and BK-channels might require fully functional c-PLA_2, we repeated the IP protocol using cells that had been treated with either AACOCF3 (15 μM), a competitive inhibitor, or aristolochic acid (250 μM), a non-competitive inhibitor [9,24]. Lanes 3 and 4 in Fig. 1 show that, even in the presence of inhibitors, c-PLA_2 and BK-channels could associate. We also reversed the order of the immunoprecipitation: immunoprecipitating with an antibody to the α-subunit of BK-channels. After solubilizing the precipitate and resolving the precipitated proteins on an SDS–gel, we used a commercially available antibody and detected the c-PLA_2 (data not shown). In an attempt to determine whether this association occurred at the membrane surface, we labeled the membrane proteins from GH3 cells with biotin and precipitated the biotin-labeled proteins by exposure to avidin beads. After releasing the membrane proteins from beads, we immunoprecipitated these proteins with c-PLA_2. Following solubilization of the precipitate and resolution on an SDS–gel, we used the α-BK-channel antibody to detect the presence of BK-channels. Fig. 2 shows that c-PLA_2 and BK-channels are indeed associated at the cell surface.

Immunohistochemistry demonstrates c-PLA_2 and BK-channels are closely associated at the cell membrane

The hypothesis that BK-channels and c-PLA_2 are closely associated was further tested by immunohisto-

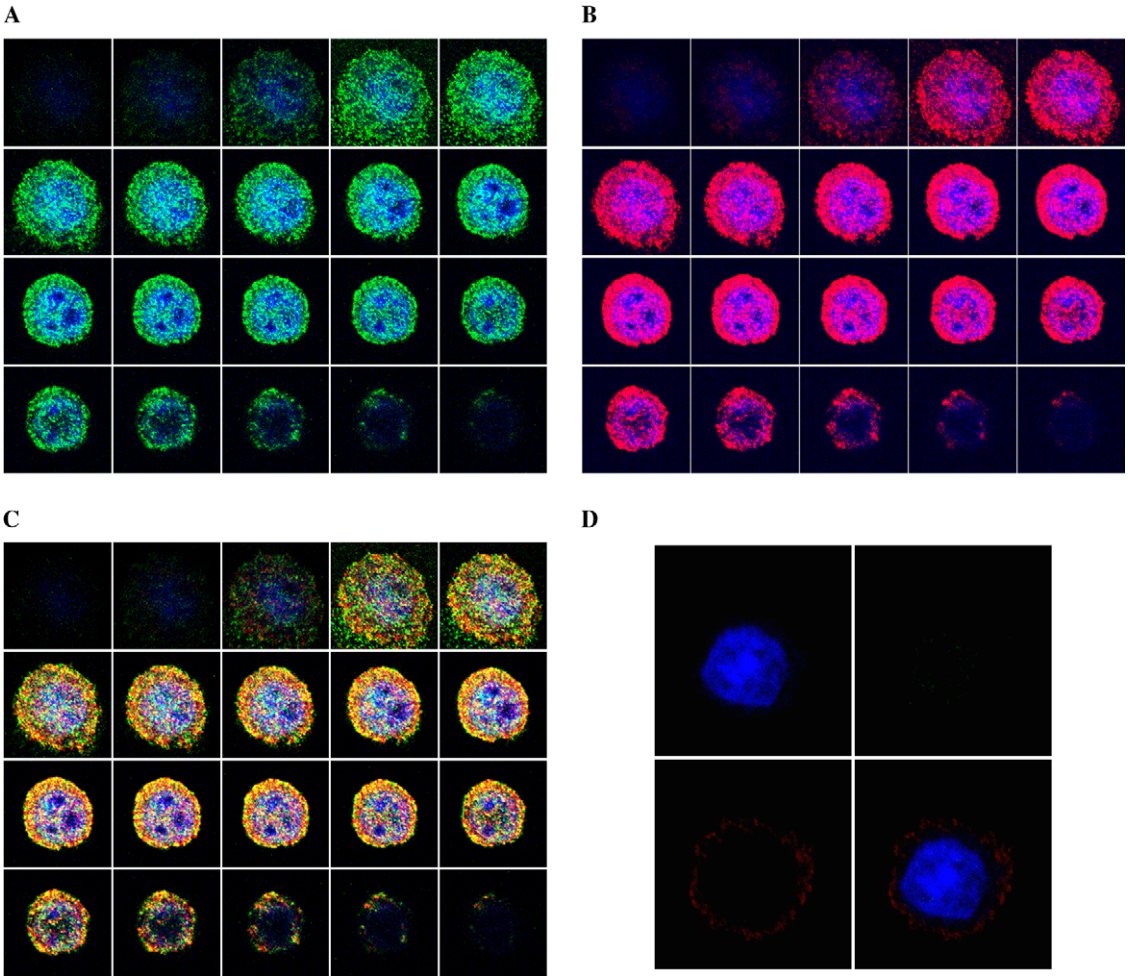

Fig. 5. c-PLA_2 and BK-channels are ubiquitously distributed throughout the cell but closely associated when both are at the plasma membrane in GH3 cells. As described for Figs. 3 and 4, GH3 cells were stained with primary antibodies directed toward c-PLA_2 (host: mouse) and the α-subunit of BK-channels (host: rabbit). Following treatment with TO-PRO-3® (blue nuclear stain) and fluorescent secondary antibodies c-PLA_2 (red) and BK-channel (green) the cells were examined using confocal microscopy using a Zeiss LSM 510 NLO META confocal microscope. In an effort to more clearly illustrate the changes in distribution and association of c-PLA_2 and α-BK, the twenty images in (A–C) are sequential 0.25 μ slices from the bottom to the top of a single GH3 cell. (A,B) Reveal the presence, BK and c-PLA_2 channel proteins, respectively, while (C) is the composite image. The yellow pixels in the composite image demonstrate the close association of the two proteins (a result supported by the co-immunoprecipitation in Fig. 1). (D) is the negative control stained with TO-PRO-3® and the two secondary antibodies showing a small amount of non-specific binding. To visualize the fluorescence in the negative control, the amplified gain had to be increased approximately 10-fold over that used to obtain images in (A–C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

chemistry. GH3 cells were fixed with paraformaldehyde and treated with a c-PLA_2 (mouse) and a BK-channel (rabbit) antibody. The cells were then treated with different fluorescent secondary antibodies. Following confocal microscopy, image analysis revealed that there was diffuse and discrete (red and green) distribution of both proteins throughout the cytosol, but the c-PLA_2 and BK-channel proteins actually at the plasma membrane were co-localized as evidenced by a composite yellow color (see Figs. 3–5). We conclude that BK-channel proteins are likely physically associated with c-PLA_2 at the cell surface. In previous work, we showed that c-PLA_2 was located in the cytosol as well as the cell membrane in unstimulated GH3 cells [25]. To test the hypothesis that most BK-channels were associated with c-PLA_2 in untreated cells, we examined the co-localization of BK and c-PLA_2 fluorescence using a quantitative algorithm (co-localization plugin in the Image J program; see Methods). Fig. 6 shows a series of slices from two typical cells that indicates co-localization (in white) in most of the slices and the scatter plots for the pixel intensities in the red and green channels. Fig. 7 shows a slice from the middle of the confocal stack for two cells. These slices emphasize that a large fraction of the pixels which show high intensities of both BK and c-PLA_2 fluorescence (indicated in white) are associated with the surface membrane of the cells (although there is some co-localization at other places in the cell, possibly the endoplasmic reticulum). When we further quantified the co-localization (Table 1), we found that the overlap coefficients for all the cells (84 slices from 15 cells) we examined were very high (0.926 ± 0.00133) and the relative intensities of green and red in the overlapping pixels were not significantly different. On the other hand, the percentage of overlapping, high intensity pixels made up a relatively small fraction of the total pixels per slice ($8.620\% \pm 0.948\%$), consistent with the relatively discrete distribution of the co-localized pixels.

As an indication that the association was likely to be physiologically relevant, we examined the localization of c-PLA_2 in GH3 cells pre-treated with the calcium ionophore A23187 which stimulates translocation and activation of c-PLA_2 from the cytosol to the plasma membrane [18]. Fig. 8 shows a marked decrease in c-PLA_2 in the

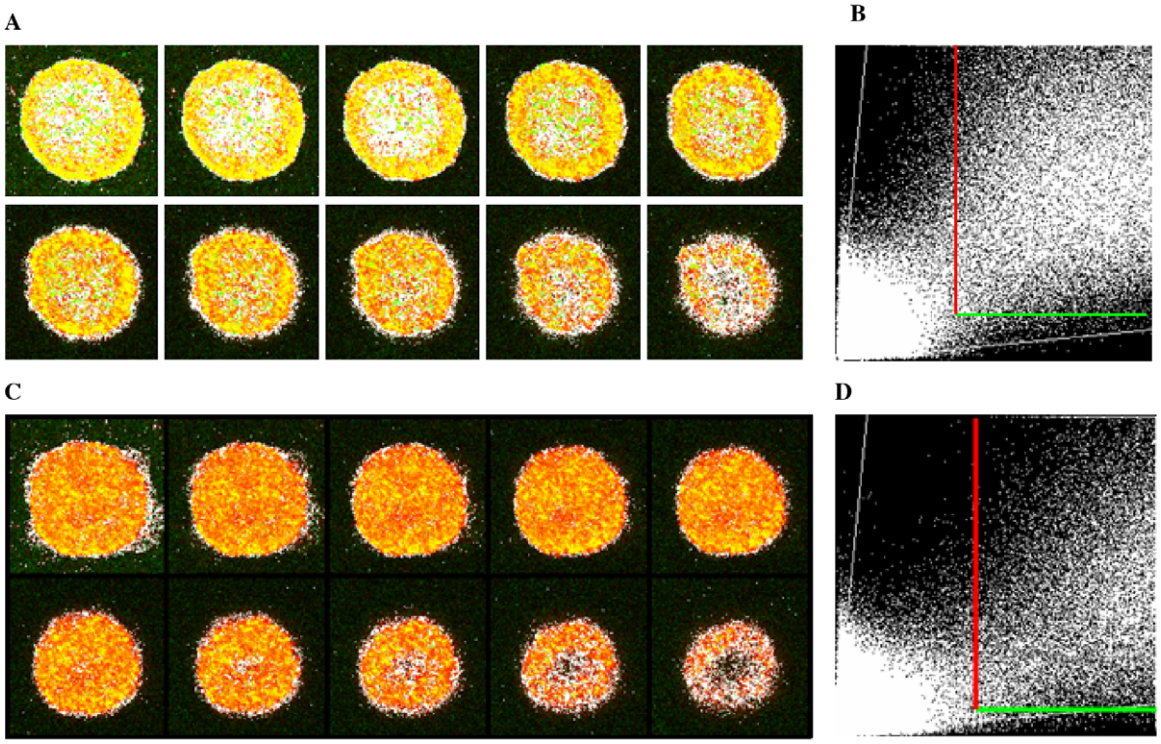

Fig. 6. Co-localization of BK-channels and c-PLA_2. As described for previous immunohistochemical figures, GH3 cells were stained with primary antibodies directed toward c-PLA_2 (host: mouse) and the α-subunit of BK-channels (host: rabbit). Following treatment with TO-PRO-3® (blue nuclear stain) and fluorescent secondary antibodies c-PLA_2 (red) and BK-channel (green) the cells were examined using confocal microscopy using a Zeiss LSM 510 NLO META confocal microscope. Ten sequential optical slice images at 0.25 μ intervals were analyzed for co-localization of red and green pixels using a quantitative algorithm (co-localization Finder plugin in the Image J program; see Methods). (A,C) A series of slices from two typical cells that indicate co-localization (in white) in most of the slices. To the right (B,D) are typical intensity scatter plots (in this case for the fifth slice from each cell) for the pixel intensities in the red (x-axis) and green channels (y-axis). In the scatterplot the collection of points near the origin represents background pixels with low green and red intensities. Only the pixels that have a higher intensity green and red component (above the green and red threshold lines) are used to generate the co-localized point shown in white. The other areas of the figures represent a traditional merge of the green and red channels. Yellow areas may represent additional co-localization, but the intensities in the red and green channels are lower than the white highlighted pixels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

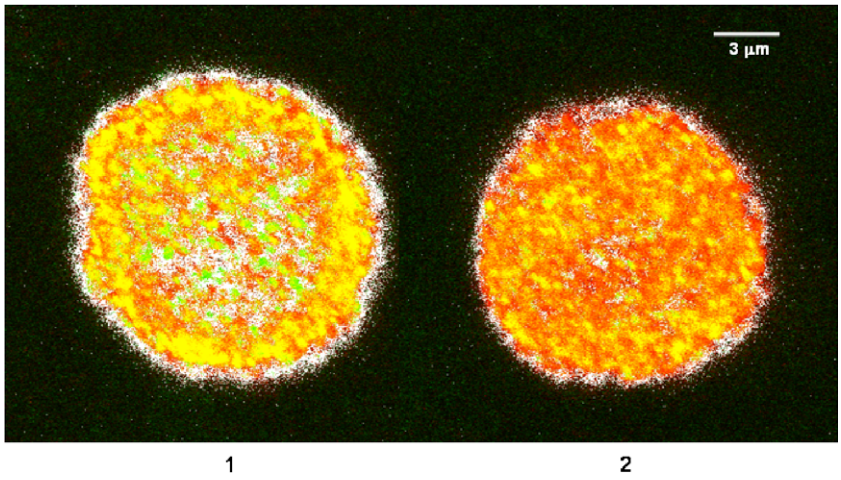

Fig. 7. BK-channels and c-PLA_2 are strongly co-localized at or near the plasma membrane of GH3 cells. This figure shows one slice from the middle of the confocal stack for two representative cells from the panels in Fig. 6. These slices emphasize that a large fraction of the pixels which show high intensities of both BK and c-PLA_2 fluorescence (indicated in white) are associated with the surface membrane of the cells (although there is some co-localization at other places in the cell, possibly the endoplasmic reticulum). These data support our findings presented in Fig. 2.

Table 1
Co-localization parameters

Pearson's correlation coefficient	0.186 ± 0.0136
Overlap coefficient (R)	0.926 ± 0.00133
BK-channel overlap coefficient (k_1)	0.907 ± 0.0270
PLA_2 channel overlap coefficient (k_2)	1.013 ± 0.0278
Slope of correlation function	0.180 ± 0.0137
Intercept of correlation function	84.4 ± 1.8
% overlapping pixels	8.620 ± 0.948
Minimum intensity BK-channel	54.2 ± 1.12
Maximum intensity BK-channel	202 ± 1.06
Minimum intensity c-PLA_2 channel	58.6 ± 0.921
Maximum intensity c-PLA_2 channel	207 ± 1.02

Values were determined according to the formulas described in Methods for 84 slices from 15 cells. Values are means ± SEM.

cytosol. There is a corresponding increase in the amount of c-PLA_2 at the membrane. Unlike Figs. 3 and 4, which show essentially no excess c-PLA_2, Figs. 8 and 9 show that there is now c-PLA_2 at the cell membrane which is not associated with a BK-channel protein. These data further support our hypothesis that in the untreated state, nearly every BK-channel is associated with c-PLA_2 but not every c-PLA_2 is associated with a BK-channel.

Discussion

For many years, the conventional wisdom taught that the sensitive relationship between activity (P_0) and Ca_i^{2+} for the entire family of Ca^{2+}-activated K^+ channels (SK, IK, and BK) was due solely to the binding of multiple Ca^{2+} to the channel protein. It was not until the report by Adelman's group that this sensitivity in SK-channels was, in fact, dependent on calmodulin rather than binding of multiple Ca^{2+} to the channel protein, itself, that this paradigm was challenged [13]. At about the same time, we reported that at least part of the Ca^{2+} sensitivity of BK-channels in GH3 cells was due to the production of arachidonic acid by c-PLA_2 residing in or near the membrane surface [14]. We reported that pharmacologic activation of c-PLA_2, e.g., by mellitin resulted in a marked increase in BK-channel activity. Conversely, any pharmacologic manipulation which prevents c-PLA_2 activation (aristolochic acid, AACOCF3) or reduces c-PLA_2 protein expression (antisense oligonucleotides) makes BK-channels markedly insensitive to intracellular calcium (although nanomolar concentrations of Ca^{2+} are required to maintain the integrity of the channels) [14]. Since these single channel data were obtained using the excised (inside out) patch configuration of the voltage clamp technique, c-PLA_2 and BK-channel proteins at the cell surface must be either in extremely close proximity or actually physically associated. We first tested this hypothesis using co-immunoprecipitation followed by Western blotting. The α subunit of the BK-channel was strongly co-immunoprecipitated with the c-PLA_2, suggesting that the BK-channel α subunits are associated with c-PLA_2. Since it has been reported that immunoprecipitation of BK-channel α-subunits results in the co-precipitation of the corresponding β-subunit, we cannot state unequivocally that c-PLA_2 is associated with the α-subunit. It is possible that c-PLA_2 associates with both subunits or with the tetrameric channel structure [20]. In addition, the association of c-PLA_2 and BK-channels can apparently occur whether c-PLA_2 is active or not since the c-PLA_2 and BK-channels still associated in the presence of both competitive and non-competitive inhibitors of c-PLA_2.

There is disagreement in the literature regarding association of c-PLA_2 with the plasma membrane. Some groups claim that in the absence of Ca^{2+}-induced translocation, very little of the c-PLA_2 pool resides at or near the plasma membrane. On the other hand, evidence of plasma membrane association in the absence of Ca^{2+} stimulation has

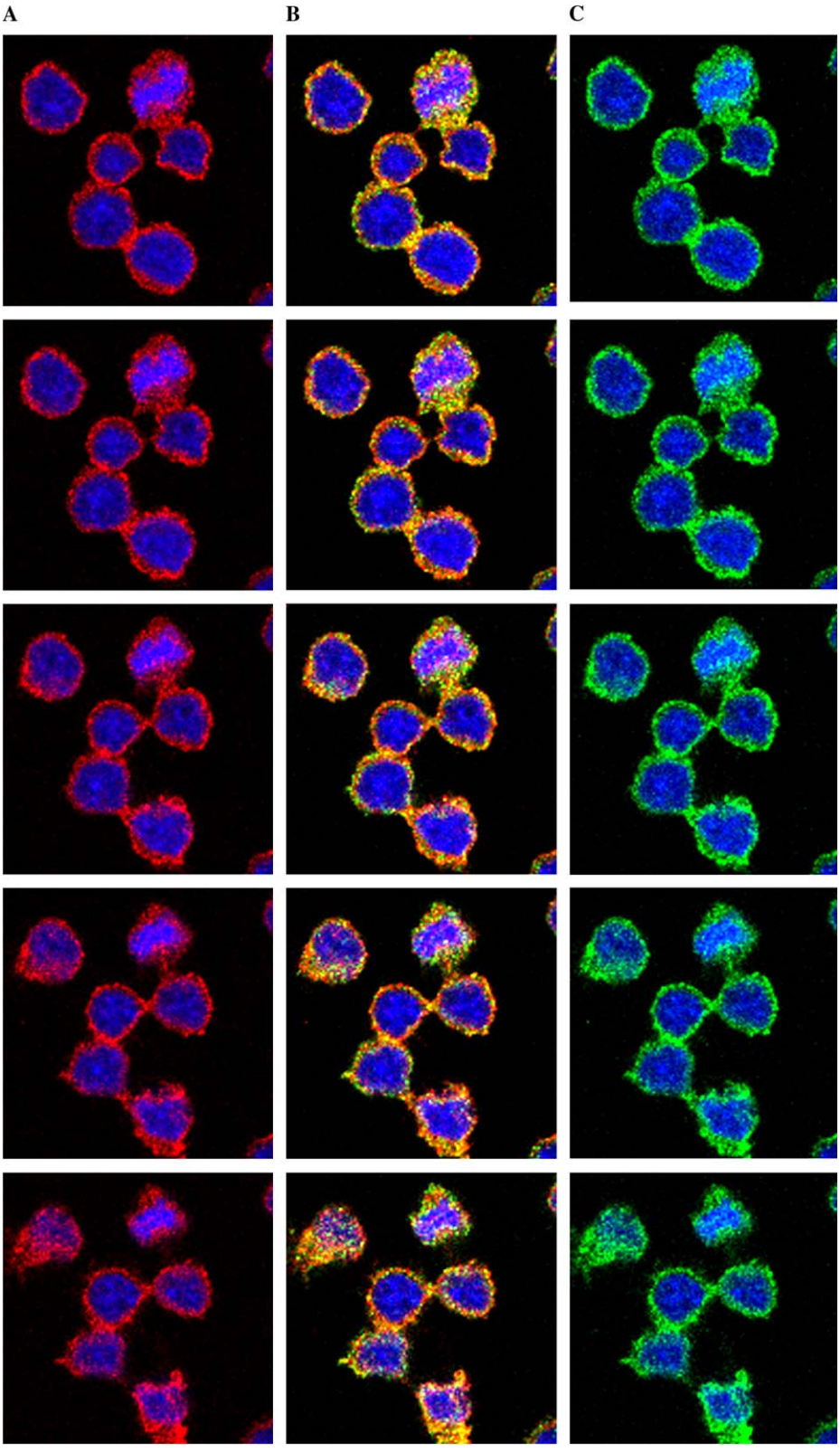

Fig. 8. A23187 results in translocation of c-PLA2 to the plasma membrane in GH3 Cells. Following treatment with A23187 as described in Methods, GH3 cells were stained with primary antibodies directed toward c-PLA_2 (host: mouse) and the α-subunit of BK-channels (host: rabbit). Following treatment with TO-PRO-3® (blue nuclear stain) and fluorescent secondary antibodies c-PLA_2 (red) and BK-channel (green) the cells were examined using confocal microscopy using a Zeiss LSM 510 NLO META confocal microscope. The five images in (A–C) are sequential 0.25 μ slices near the middle of the cells. (A,C) Reveal the presence of c-PLA_2 and BK-channel proteins, respectively, while (B) is the composite image. The yellow pixels in the composite image demonstrate the close association of the two proteins. It is also apparent that there is essentially no c-PLA2 remaining in the cytosol and it is essentially all at the plasma membrane. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

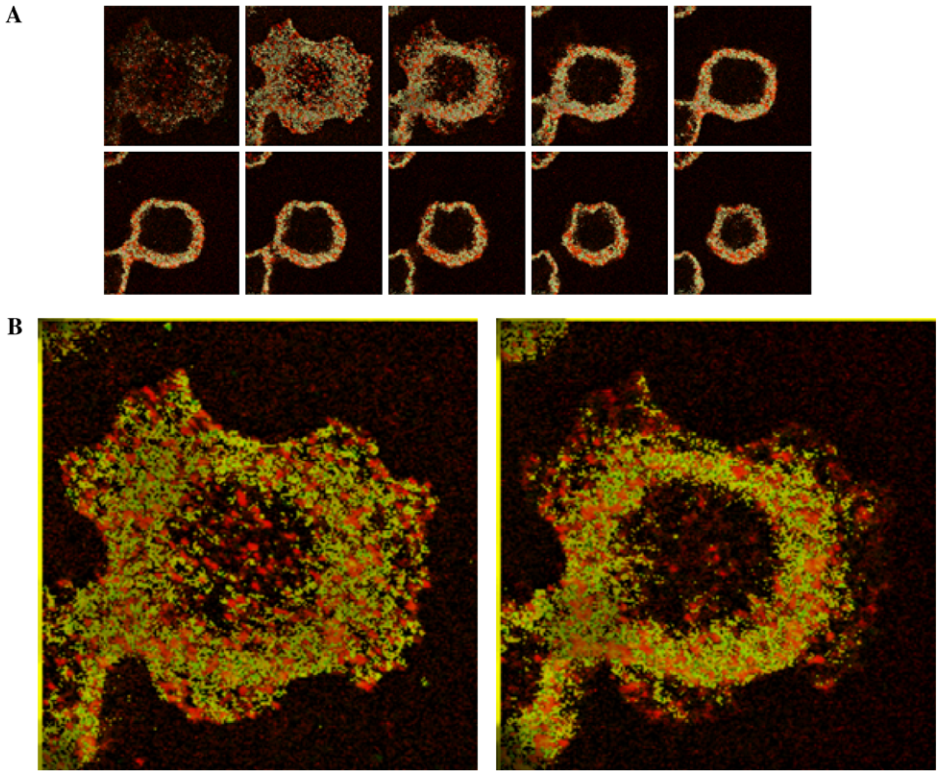

Fig. 9. After activation with A23187, c-PLA2 is no longer strongly associated with BK-channels in GH3 Cells. Following treatment with A23187 as described in Methods and in the legend of Fig. 6, we analyzed the association of c-PLA_2 with BK-channels. A23187 increases intracellular calcium and promotes activation and translocation of c-PLA_2. We created images in which co-localization of BK and c-PLA_2 and BK-channels by themselves is indicated in green and c-PLA_2 that is not co-localized with BK-channels is indicated in red. In (A) are 10 sequential 0.25 μ slices from near the bottom (growth substrate side) of the cells upward towards the top surface. In (B) are two typical slices from the cell (below the nucleus). Both panels show significant amounts of unassociated c-PLA_2 that is not seen in the absence of A23187 (Figs. 6 and 7), indicating that BK-channels are usually associated with c-PLA_2, but c-PLA_2 is not obligatorily associated with BK-channels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

been reported for a number of cell types [18]. If c-PLA_2 were not present in the cell membrane of untreated GH3 cells, then it would be difficult to reconcile with our observation that c-PLA_2 activity appears to be constitutively associated with the small patches of plasma membrane excised with our patch pipettes. An explanation of this apparent discrepancy involves the observation by Osterhout and Shuttleworth that at least a portion of the cellular pool of c-PLA_2 resides in the cell membrane and appears to be activated without increases in $[Ca_i^{2+}]$ [26]. Our previously reported data showing that there is a significant amount of c-PLA_2 in the cellular membranes of untreated GH3 cells are consistent with this observation [25]. This observation has now been further validated by immunohistochemistry. Following confocal microscopy, image analysis revealed that the c-PLA_2 and BK-channel proteins at the plasma membrane were co-localized as evidenced by a composite yellow color and by co-localization analysis. Experiments with A23187 show a marked decrease in c-PLA_2 in the cytosol and a corresponding increase in c-PLA_2 at the cell membrane. Under these conditions, the c-PLA_2 in the surface membrane does not appear to be as strongly associated with BK-channels. We conclude that while c-PLA_2 and BK-channels are ubiquitously distributed throughout the cell and only weakly associated (possibly in the endoplasmic reticulum prior to trafficking to the membrane), BK-channels in the plasma membrane of untreated GH3 cells are strongly associated with c-PLA_2 and therefore, as also suggested by the co-immunoprecipitation, are likely physically associated with c-PLA_2. On the other hand, while BK-channels are strongly associated with c-PLA_2, some c-PLA_2 molecules do not appear to be associated with a BK-channel. This observation may not be surprising since the cellular concentration of c-PLA_2 is high compared to BK-channels and it subserves other roles in the cell besides acting as a modulator of BK-channel activity.

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