Cytosolic Phospholipase A$_2$ Is Required for Optimal ATP Activation of BK Channels in GH$_3$ Cells*

To test the hypothesis that ATP activation of BK channels in GH$_3$ cells involves cytosolic phospholipase A$_2$ (cPLA$_2$) as a potential protein target for phosphorylation, we first inhibited the activity of cPLA$_2$ by both pharmacologic and molecular biologic approaches. Both approaches resulted in a decrease rather than an increase in BK channel activity by ATP, suggesting that in the absence of cPLA$_2$, phosphorylation of other regulatory elements, possibly the BK channel protein itself, results in inactivation rather than activation of the channel. The absence of changes in activity in the presence of the non-substrate ATP analog 5'-adenylyl-β,γ-imidodiphosphate verified that ATP hydrolysis was required for channel activation by ATP. Experiments with an activator and inhibitor of protein kinase C (PKC) support the hypothesis that PKC can be involved in the activation of BK channels by ATP; and in the absence of PKC, other kinases appear to phosphorylate additional elements in the regulatory pathway that reduce channel activity. Our data point to cPLA$_2$-α (but not cPLA$_2$-γ) as one target protein for phosphorylation that is intimately associated with the BK channel protein.

Long-term modulation of ion channels is beginning to be appreciated. This concept suggests that changes in ion channel properties are not dependent on continued occupation of a receptor by an agonist, but rather arise via some long-lasting metabolic modification such as protein phosphorylation (1–3). For this process to occur, the receptor and ion channel do not have to be intimately associated, but communicate some signal transduction pathway activated by occupancy of the receptor. Such a signal transduction pathway, which may involve several steps, ultimately results in a modification that alters the activity and persists until this modification is reversed (1). Protein phosphorylation and dephosphorylation have been shown to be important in the modulation of a number of ion channels, particularly those in the central nervous system (1–4). One important type of central nervous system ion channel that has been shown to be modulated by phosphorylation/dephosphorylation is the large conductance Ca$^{2+}$-activated potassium (BK) channel, with at least six functionally distinct types having been described within the central nervous system (5). BK channels are important to such widely diverse central nervous system functions as neural regulation of the heart originating in the nucleus tractus solitarius and sleep, which is dependent on repetitive rhythmic activity originating in the reticular formation of the thalamus (6–8). BK channels are also involved in neuropeptide secretion, regulation of presynaptic calcium signals, and neurotransmitter release (9). Because of the physiologic importance of BK channels, we have been particularly interested in the cellular signaling mechanisms responsible for controlling the activity of BK channels (10, 11).

It is well documented that BK channels that have been reconstituted in lipid bilayers can be either activated or inhibited by the addition of ATP, protein kinases, or protein phosphatases (1, 4, 9, 12, 13). In fact, BK channels have been classified as either type I or II based on their response to the catalytic subunit of the cAMP-dependent protein kinase (4). Type II BK channels in mammalian brain reconstituted in lipid bilayers are activated by ATP and ATP analogs via an endogenous protein kinase activity intimately associated with the channel. For these channels, it appears the kinase involved is protein kinase C (PKC)$^\dagger$ since activators of PKC enhance the response to ATP, whereas inhibitors of PKC reverse the response to ATP (1). BK channels in GH$_3$ cells appear to belong to the class that is activated in the presence of ATP with or without the addition of exogenous protein kinase, whereas BK channels in GH$_4$ cells appear to be inactivated by ATP or protein kinases and activated by protein phosphatases (14, 15). Investigators examining BK channels that are activated in response to ATP in lipid bilayers concluded that there must be an endogenous protein kinase activity intimately associated with these channels (1, 4, 9, 13). However, because BK channels in lipid bilayers are at infinite dilution, the phosphorylation target could be either the channel protein itself or a regulatory protein that is intimately associated with the ion channel. The possibility that the action of the kinase may be on a regulatory protein rather than (or in addition to) the channel protein itself is intriguing. One important regulatory protein for BK channels in GH$_3$ cells is cytosolic phospholipase A$_2$ (cPLA$_2$) (10, 11). Until recently, it has been thought that optimal activation of cPLA$_2$ requires both Ca$^{2+}$ and phosphorylation (16–19). It was also thought that cPLA$_2$ resides in the cytosol and translocated to the cell membrane only in response to ATP or protein kinases.

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The abbreviations used are: PKC, protein kinase C; cPLA$_2$, cytosolic phospholipase A$_2$; ATPγS, adenosine 5′-O-(thiotriphosphate); AMP-PPN, 5′-adenylyl-β,γ-imidodiphosphate; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate.
to large increases in $[\text{Ca}^{2+}]_i$. It is now clear that the mechanisms involved in the regulation of cPLA$_2$ include the relative importance of phosphorylation, $[\text{Ca}^{2+}]_i$, and the site of subcellular localization, show considerable variability in different cell types and even between different agonists in the same cell type. However, in most cells, there is a significant pool of cPLA$_2$ constitutively associated with the membrane capable of producing arachidonic acid (17, 20). In addition, recent evidence suggests that there is more than one isoform of cPLA$_2$ (21) that responds to intracellular calcium and other stimuli differently.

In this investigation, we sought to determine whether cPLA$_2$ could be a potential target for phosphorylation, resulting in subsequent activation of BK channels. To test this hypothesis, we studied the response of wild-type BK channels in GH$_3$ cells to ATP, to the poorly hydrolyzable analog ATP-γS, and to the non-hydrolyzable substrate AMP-PNP. These results were compared with responses from BK channels that had been exposed to either aristolochic acid or antisense oligonucleotides to cPLA$_2$ prior to the addition of ATP. Since PKC has been reported to be involved in the activation of reconstituted BK channels as well as the phosphorylation of cPLA$_2$, we examined the possibility that PKC could be associated with the ATP response of BK channels in GH$_3$ cells. Finally, since two isoforms of cPLA$_2$, viz. cPLA$_2$-α (86 kDa) and cPLA$_2$-γ (60 kDa), have now been identified, we also sought to verify that the cPLA$_2$-α isoform is the one closely associated with BK channels in GH$_3$ cells (21).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The GH$_3$ cell line was obtained from American Type Culture Collection (Manassas, VA). GH$_3$ cells were grown at 37 °C in a 5% CO$_2$ atmosphere in Dulbecco's modified Eagle's medium supplemented with 15% heat-activated horse serum, 2.5% fetal bovine serum, and 2 mM glutamine. Cells for electrophysiologic experiments were plated on polylysine-coated Petri dishes to which a polycarbonate recording chamber with a volume of 0.2 ml had been previously affixed with Sylgard$^\text{TM}$. Cells were used 1–3 days after plating, and cells from passages 23 to 40 were used in the experiments described in this study.

**cPLA$_2$ Sense and Antisense Oligonucleotides**—Sense and antisense oligonucleotides targeting two possible translation start codons of rat cPLA$_2$ were synthesized by the Microchemical Facility of Emory University as previously reported (11). A GenBank$^\text{TM}$/EBI Data Bank search using the sequences for the sense and antisense oligonucleotides used in this investigation resulted in only one full match, viz. cPLA$_2$-α, and no partial matches. Since inhibition by antisense oligonucleotides usually requires a perfect match between oligonucleotide and target mRNA, it is extremely unlikely that our antisense oligonucleotides bound to cPLA$_2$-γ mRNA (if it were present) or to any other mRNA present in GH$_3$ cells.

**Western Blot Analysis**—Approximately 10$^5$ cells were washed twice with phosphate-buffered saline without Ca$^{2+}$ or Mg$^{2+}$. Cells were lysed at 4 °C in the buffer described by Leslie and co-workers (17). Following cell lysis, the suspension was centrifuged at 2000 $\times$ g for 10 min to precipitate unlysed cells. Protein determinations on the supernatant were accomplished using a commercial protein assay kit (Bio-Rad DC Protein Assay Kits$^\text{TM}$). Absorbance measurements were made on an Ultrorad$^\text{TM}$ 3000 (Amersham Pharmacia Biotech). Samples were prepared for SDS-polyacrylamide gel electrophoresis by diluting cell lysate with sample buffer (Tris (pH 6.8), 0.1% SDS, 10% glycerol, and 0.025% bromophenol blue) and heating at 85 °C for 2 min. 30 μg of protein were loaded in each lane on a 7.5% polyacrylamide gel. Following electrophoresis (~1 h at 150 V), the proteins were transferred to nitrocellulose. The nitrocellulose blots were then probed with a specific anti-cPLA$_2$ antibody (a generous gift from Dr. Ruth Kramer (Lilly) following the protocol provided by Amersham Pharmacia Biotech for ECL developing). Detection of the resulting colonies using an alkaline extraction miniprep kit (QIA-GEN Inc.) and sequenced at the Emory University Sequencing Facility.

**Drug Exposure Paradigm**—For all experiments, drug exposure was essentially using a gravity infusion/suction system that was controlled both by a peristaltic pump and a gravity feed. Each reaction mixture contained 2 μM Ca$^{2+}$ and either 2 mM MgATP or 200 μM ATPγS, and recordings were continuously made for 10–15 min. For experiments involving aristolochic acid, the patch was initially exposed to 0.1 μM Ca$^{2+}$, and after control recordings (typically 2–5 min), the patch was perfused with a second solution containing 0.1 μM Ca$^{2+}$, and either 2 mM MgATP or 200 μM ATPγS, and recordings were continuously made for 10–15 min. For experiments involving aristolochic acid, the patch was initially exposed to either 0.1 or 1 μM Ca$^{2+}$. After control recordings (typically 2–5 min), the patch was perfused with a solution containing 250 μM aristolochic acid, and recordings were made for 10 min. This paradigm was then repeated with either 2 mM MgATP or 200 μM ATPγS, and recordings were continued for an additional 10–15 min. All solutions containing ATP, ATPγS, phorbol esters, or GF 109203X were made immediately prior to use.

**Electrophysiologic Recordings**—For electrophysiologic measurements, cells were gently suspended in phosphate-buffered saline following cPLA$_2$ treatment. Single channel experiments were conducted with the patch depolarized to +20 mV. Single channel data were stored on digital audio tape using a Sony Model DAS-75 digital audio tape recorder (Dagan Corp., Minneapolis, MN).

**Data Analysis**—Single channel data were digitized using Axotape software (Axon Instruments, Inc., Foster City, CA) at a sampling rate of 5 kHz and filtered at 2 kHz using a four-pole low-pass Bessel filter. The digitized single channel data were analyzed in 1-min segments to generate $N_P$ versus time plots using Fetchan and P-Stat software programs (Axon Instruments, Inc.). $N_P$ versus time plots were used to determine the time course for reaching a stable maximum effect for each series of experiments. Open probabilities were determined from the amplitude histograms by fitting each amplitude histogram to the appropriate sum of gaussian distribution functions using iterative non-linear regression software (PeakFit Version 4, SPSS Inc., Chicago, IL) after correction of the baseline to make zero current coincident with the state in which all channels were closed. $N_P$ values were first calculated from the amplitude histogram. The open probability ($P_o$) was then calculated as $N_P/N$. The number of channels in each patch ($N$) was calculated by dividing the amplitude probability histogram by the probability density function of the patch to 10 or 100 μM Ca$^{2+}$, by the unit conductance associated with a single-channel state. Because the duration of open and closed intervals varied from very short to very long durations, data obtained from a patch that contained a single channel were binned logarithmically and analyzed according to the method of Sigworth and Sine (25).

**Solutions**—The solutions used in all experiments were as follows:

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RESULTS

BK Channels in GH3 Cells Are Activated by MgATP and ATPγS, but Not by AMP-PNP—All of these experiments were done using the excised patch configuration of the voltage-clamp technique with the patch depolarized to +20 mV. Both the bath and pipette solutions contained 140 mM K+, and the bath solution also contained 0.1 mM Ca2+. After control recordings had been made, 1 mM MgATP, 200 mM ATPγS, or 1 mM AMP-PNP (a non-substrate analog) was added, and recordings were continued until any effects had stabilized. MgATP and ATPγS caused the Po to increase significantly in all cells studied (p < 0.001) (Fig. 1A and B). MgATP caused the Po to increase from 0.14 ± 0.06 to 0.19 ± 0.07, whereas ATPγS resulted in an increase in Po from 0.10 ± 0.04 to 0.24 ± 0.10. The magnitude of the effect noted for ATPγS was significantly larger than that noted for MgATP (p < 0.001) presumably because there was a phosphorylation/dephosphorylation equilibrium established in the MgATP experiments, whereas the reaction of ATPγS is only poorly reversible. On the other hand, AMP-PNP (Fig. 1C) did not result in any significant change in Po (0.14 ± 0.02 versus 0.14 ± 0.03), suggesting that the effects of ATP were not simply ligand-stimulated increases in channel activity.

Aristolochic Acid Can Either Inhibit or Reverse the Activation of BK Channels by MgATP—Because cPLA2 is an important element in the regulation of BK channels in GH3 cells and since cPLA2 can be activated by phosphorylation, we examined the effects of MgATP in the presence of the pharmacologic cPLA2 inhibitor aristolochic acid. All of the following experiments were done using the excised patch configuration of the voltage-clamp technique with the patch depolarized to +20 mV. Both the bath and pipette solutions contained 140 mM K+, and the bath solution also contained 1 µM Ca2+. Two sets of experiments were conducted. First, after control single channel recordings had been made on excised patches, a solution containing 250 µM aristolochic acid was introduced. After a 5-min exposure, single channel recordings were again made. Finally, a solution containing both 250 µM aristolochic acid and 1 mM MgATP was introduced, and recordings were continued for an additional 10 min. (The second set of experiments started with 1 mM MgATP being added to the bath solution after control single channel recordings had been made.) After the effects of MgATP had stabilized, a solution containing 1 mM MgATP and 250 µM aristolochic acid was introduced, and single channel recordings were made for an additional 10 min. In the first set of experiments, the addition of aristolochic acid caused a significant reduction in Po from 0.36 ± 0.05 to 0.06 ± 0.04 (p < 0.001). Subsequent addition of MgATP caused the Po to change from 0.06 ± 0.04 to 0.04 ± 0.03. These data are summarized in Fig. 2A. In the second set of experiments, the addition of MgATP resulted in a significant increase in Po from 0.15 ± 0.05 to 0.22 ± 0.06. Subsequent addition of aristolochic acid resulted in a significant decrease in Po to 0.05 ± 0.03 (p < 0.001). These data suggested that cPLA2 was a target for phosphorylation by ATP.

Treatment of Cells with cPLA2 Antisense Oligonucleotides Eliminates the Activation of BK Channels by MgATP—Because aristolochic acid is a pharmacologic inhibitor of cPLA2 and could possibly be producing its effect on the MgATP response via some other mechanism, we examined the effects of MgATP on excised patches derived from cells treated with cPLA2 antisense oligonucleotides. For the following experiments, the excised patch configuration of the voltage-clamp technique was used with the patch depolarized to +20 mV. Both the bath and pipette solutions contained 140 mM K+, and the bath solution also contained 1 µM Ca2+. After single channel recordings for antisense oligonucleotide-treated cells had been made, the patches were exposed to 1 mM MgATP, and recordings were continued for 10 min. The Po decreased from 0.13 ± 0.06 to 0.08 ± 0.06 (p < 0.05) following treatment with MgATP. These data are presented in Fig. 2B. Both biochemical and Western blot analyses verified that the expression of cPLA2 had been
reduced by >90% from control levels, as we have previously reported (11). These data further support the hypothesis that ATP phosphorylation of cPLA2 is an important pathway for the activation of BK channels by ATP.

PKC Is Involved in the Activation of BK Channels by ATP—PKC (or PKC-like kinase) activity has been reported to be associated with BK channels that have been reconstituted in lipid bilayers. It is likely that such kinase activity could be preserved in excised patches containing BK channels. To test this hypothesis, we conducted three sets of experiments. For all of the following experiments, the excised patch configuration of the voltage-clamp technique was used with the patch depolarized to +20 mV. Both the bath and pipette solutions contained 140 mM K+, and the bath solution also contained 1 μM Ca²⁺, for wild-type cells and 1 μM Ca²⁺, for experiments in which cells treated with cPLA2 antisense oligonucleotides were used.

In the first set of experiments, after control single channel recordings had been made, the patches were exposed to 1 mM MgATP, and recordings were continued for 10 min. After the effects had stabilized, a solution containing 1 mM MgATP and either 1 μM PMA or α-phorbol (as an inactive control) was added, and recordings were made for an additional 10 min. The addition of 1 mM MgATP resulted in a significant increase in \( P_o \) from 0.22 ± 0.04 to 0.29 ± 0.04 (\( p < 0.001 \)). Subsequent addition of PMA resulted in a further significant increase in \( P_o \) to 0.41 ± 0.04 (\( p < 0.001 \)) (Fig. 3A). On the other hand, the addition of α-phorbol did not result in any additional increase in \( P_o \) over the increase resulting from MgATP. In the second set of experiments, the above paradigm was repeated on cells that had been treated with cPLA2 antisense oligonucleotides. As expected from experiments described earlier, the addition of MgATP resulted in little change in \( P_o \) from control levels. The \( P_o \) for control conditions was 0.10 ± 0.04 and 0.08 ± 0.05 after treatment with MgATP. The addition of PMA also produced little change, with a \( P_o \) following treatment with PMA of 0.079 ± 0.06 (Fig. 3B). In the third set of experiments, after control single channel recordings had been made, 20 μM GF 109203X, a potent PKC inhibitor, was introduced, and recordings were continued for 10 min. A solution containing both GF 109203X and 1 mM MgATP was then introduced, and recordings were again made for 10 min. Finally, the patches were exposed to a solution once again containing only GF 109203X, and recordings were made for a final 10 min. The addition of GF 109203X did not result in a significant change in \( P_o \) from control levels (0.27 ± 0.03 to 0.28 ± 0.03). However, the addition of 1 mM MgATP in the presence of GF 109203X caused the \( P_o \) to significantly decrease from 0.28 ± 0.03 to 0.17 ± 0.06 (\( p < 0.005 \)). The washout of MgATP resulted in a significant increase (\( p < 0.0025 \)) and restored the \( P_o \) to a control value of 0.28 ± 0.02. Similar results were obtained in three cells using the PKC pseudosubstrate inhibitor residues 19–31. These data further support the hypothesis that PKC can be involved in the activation of BK channels by ATP and that, in the absence of PKC, other kinases appear to phosphorylate additional elements in the regulatory pathway that reduce channel activity (Fig. 3C).

cPLA₂ Is Associated with Membranes in GH₃ Cells—A PLA₂ assay (described under Experimental Procedures) was conducted on both the cytosolic and membrane fractions of GH₃ cell lysates prepared using the procedure described by Osterhout and Shuttleworth (26). In wild-type GH₃ cells, 81 ± 19% of the PLA₂ was found in the membrane fraction, which was significantly greater than the 19 ± 5% found in the cytosolic fraction (\( p < 0.01 \)) (Fig. 4). It should be noted that although the PLA₂ assay employed here does not distinguish between secretory and cytosolic PLA₂, the Western blot analysis and PLA₂ amplification data described earlier suggest that cytosolic PLA₂ is the major form of the enzyme present in GH₃ cells. These data support the idea that, in GH₃ cells as well as other cells (first suggested by Osterhout and Shuttleworth (26)), a portion of the PLA₂ pool resides in the cell membrane.

cPLA₂-α Appears to Be the Major Isoform Found in GH₃ Cells—Western blot analysis of GH₃ cell lysates revealed two bands at ~86 kDa that were the phosphorylated and unphosphorylated forms of cPLA₂-α. There was no band in the 60-kDa range, which would be the size expected for cPLA₂-γ, a cPLA₂ isozyme usually found in skeletal muscle, but not in brain. To confirm that there was only cPLA₂-α and no cPLA₂-γ in GH₃ cells, we designed and synthesized PCR primers that would amplify both isoforms. Amplification of cDNA derived from GH₃ cell mRNA produced products that ran as a single 606-
Although we repeated the amplification four times, we never observed any PCR product that corresponded to the γ-isof orm and therefore concluded, as others have reported (21), that cPLA₂-α and cPLA₂-γ is not present in brain-derived cells like GH₃.

**DISCUSSION**

The major findings in this investigation are as follows. 1) ATP results in a significant activation of BK channels in wild-type GH₃ cells. 2) Inhibition of cPLA₂ by aristolochic acid blocks the activation of BK channels by ATP in wild-type GH₃ cells. 3) Reducing the expression of cPLA₂ with antisense oligonucleotides also suppresses the activation of BK channels by ATP. 4) Activation of PKC results in a significant potentiation of the activation in BK channels produced by ATP. 5) Inhibition of PKC in wild-type GH₃ cells results in a significant decrease in BK channel activity when ATP is added. 6) Inhibition of cPLA₂ blocks the activation of BK channels by PKC. 7) cPLA₂-α appears to be the isof orm associated with BK channels in GH₃ cells.

Protein phosphorylation and dephosphorylation are important in the modulation of central nervous system BK channels. (1–4). However, modulation of BK channels by phosphorylation is complicated. BK channels reconstituted in lipid bilayers can be either activated or inhibited by the addition of ATP, protein kinases, or protein phosphatases (1, 4, 12). In fact, BK channels have been classified as either type I or II based on their response to phosphorylation (4). Type II BK channels in mammalian brain reconstituted in lipid bilayers are activated by ATP and ATP analogs via an endogenous protein kinase activity intimately associated with the channel. For these reconstituted channels, it appears that the kinase involved is similar to protein kinase C since activators of PKC enhance the response to ATP, whereas inhibitors of PKC reverse the response to ATP (1, 4, 9, 13). Besides a PKC-like protein, Levitan and co-workers (27–29) have shown a close association between BK channels and a several other regulatory proteins that directly alter BK activity. BK channels in GH₃ cells appear to belong to the class that is activated in the presence of ATP with or without the addition of exogenous protein kinase, whereas BK channels in GH₅ cells (a subclone of GH₃ cells) appear to be inactivated by ATP or protein kinases and activated by protein phosphatases (9, 14, 15). To test this hypothesis, we studied the responses of wild-type BK channels to ATP in GH₃ cells.

**BK Channels in GH₃ Cells Are Activated by MgATP and ATPγS, but Not by AMP-PNP—MgATP and ATPγS caused the Pₒ of BK channels in wild-type GH₃ cells to increase significantly. The non-substrate ATP analog AMP-PNP did not result in any significant change in Pₒ, further suggesting that the effects produced by ATP require ATP hydrolysis for channel activation (30). This is different from the ATP-sensitive ion channels found in a variety of tissues in which ATP functions as a ligand to alter channel properties by binding reversibly to an allosteric site, but without ATP hydrolysis (30). The magnitude of the increase noted for ATPγS was significantly larger than that noted for MgATP presumably because there was a phosphorylation/dephosphorylation equilibrium established in the MgATP experiments, whereas the reaction of ATPγS is only slowly reversible (9, 13). This activation could be due to a direct kinase-mediated phosphorylation of the channel by ATP. Alternatively, since PL₃γ production of arachidonic acid is also a potent activator of BK channels in excised patches and since PL₃γ can be activated by phosphorylation, ATP activation of BK channels could be due to kinase-mediated phosphorylation of PL₃γ rather than direct phosphorylation of BK channels. Although these data clearly show that ATP hydrolysis and subsequent protein phosphorylation are required for activation.
of BK channels in GH3 cells, the target for phosphorylation is still unclear.

**Inhibition of cPLA2 Blocks the Activation of BK Channels by ATP in Wild-type GH3 Cells**—Investigators examining BK channels that are activated in response to ATP in lipid bilayers concluded that there must be an endogenous protein kinase activity intimately associated with these channels (1, 4, 9, 13). However, the phosphorylation target could be either the channel protein itself or a regulatory protein that is intimately associated with the ion channel (1, 2, 4, 9, 13). We have shown that one important regulatory protein for BK channels in GH3 cells is cPLA2-α (11). Optimal activation of cPLA2-α requires both Ca²⁺, and phosphorylation (16–19). In this investigation, we sought to determine whether cPLA2 could be a potential target for phosphorylation, resulting in subsequent activation of BK channels. To test this hypothesis, we used a pharmacologic and an antisense approach to lower cPLA2 activity in wild-type GH3 cells. Aristolochic acid caused a significant reduction in Pₚ for BK channels. This observation is consistent with our previous reports (11). Subsequent addition of MgATP caused the Pₚ to decrease, although this decrease was not statistically significant. The addition of aristolochic acid to patches in which BK channels were activated by ATP resulted in a significant decrease in activity. These data suggested that cPLA2 was one target for phosphorylation by ATP. Because aristolochic acid is a pharmacologic inhibitor of cPLA2 and could possibly be producing its effect on the MgATP response via some other mechanism, we examined the effects of MgATP on excised patches derived from cells treated with cPLA2 antisense oligonucleotides. As previously reported, treatment of wild-type GH3 cells in the present investigation with antisense oligonucleotides resulted in an ~90% decrease in the expression of cPLA2-α by both biochemical and Western blot analyses (11). Exposure of excised patches from antisense oligonucleotide-treated cells resulted in a modest, albeit significant decrease in BK channel activity. These data support the hypothesis that ATP phosphorylation of cPLA2-α is one important pathway for the activation of BK channels by ATP. Our data further suggest that, in the absence of cPLA2-α, there may be additional targets for phosphorylation that act to inhibit BK channel activity like the inhibition seen in GH3 cells (14, 15).

**PKC Is Involved in the Activation of BK Channels by ATP**—Type II BK channels in mammalian brain reconstituted in lipid bilayers are activated by ATP and ATP analogs via an endogenous protein kinase activity intimately associated with the channel. For these channels, it appears that the kinase involved is similar to protein kinase C since activators of PKC enhance the response to ATP, whereas inhibitors of PKC reverse the response to ATP (1, 4, 9, 13). Since PKC could activate cPLA2 either directly or via an alternative kinase pathway, we studied the effect of MgATP on BK channels in the presence of the specific PKC inhibitor GF 109203X. Treatment of excised patches with GF 109203X had no effect on the Pₚ of BK channels. However, the addition of MgATP to cells treated with GF 109293X resulted in a significant decrease in Pₚ. This decrease was similar to that observed in cells treated with antisense oligonucleotides, where the addition of MgATP also decreased the Pₚ. On the other hand, treatment of cells that had been exposed to MgATP with PMA, an activator of PKC, resulted in a significant increase in Pₚ. On the other hand, for BK channels in cells treated with cPLA2-α antisense oligonucleotides, exposure to PMA did not result in any significant change in Pₚ. These results suggest that 1) PKC is necessary for ATP activation of BK channels; 2) in the absence of PKC, other kinases appear to phosphorylate additional elements in the regulatory pathway that reduce channel activity; 3) cPLA2-α is partially phosphorylated and active in GH3 cells under basal conditions; and 4) cPLA2-α is a target for PKC phosphorylation. However, our data cannot exclude the possibility that mitogen-activated protein kinase also plays a role in the activation of cPLA2-α, as suggested by Leslie and co-workers (16, 31).

**cPLA2-α Rather than cPLA2-γ Appears to Be the Isoform Associated with BK Channels in GH3 Cells**—Recently, a novel membrane-associated cPLA2 isoform (cPLA2-γ) has been described (21) (in contrast to the original isoform, termed cPLA2-α). cPLA2-γ is associated with the plasma membrane, but it lacks the [Ca²⁺]-dependent phospholipid-binding domain found in cPLA2-α, so its activation is entirely [Ca²⁺]-independent. Although cPLA2-γ shares significant sequence homology with cPLA2-α, the absence of the phospholipid-binding domain reduces the size to ~60 kDa, so cPLA2-γ can be easily distinguished on Western blots from the “classical” type IV cPLA2 (cPLA2-α). Several lines of evidence argue against cPLA2-γ being the isoform associated with BK channels in GH3 cells. First, a GenBank™/EBI Data Bank search using the sequences for the sense and antisense oligonucleotides used in this study did not identify cPLA2-γ (or any other sequence besides cPLA2-α). Second, Western blots do not have a band at...
60 kDa, where cPLA₂-γ should run. Finally, PCR amplification of GH₃ cell mRNA using primers that would amplify any cPLA₂ isoforms in GH₃ cells resulted in the amplification of only cPLA₂-α. This is entirely consistent with the tissue distribution of cPLA₂-γ, which showed very little of this isoform in brain or brain-derived tissues (21). These data support our claim that the results with cells treated with antisense oligonucleotides were, in fact, due solely to the depletion of cPLA₂-α.

A Portion of the cPLA₂-α Pool in GH₃ Cells Resides in the Cell Membrane of Wild-Type Cells—Until recently, it has been thought that cPLA₂ resides almost exclusively in the cytosol and translocates to the cell membrane (and other membranes) only when stimulated by large increases in [Ca²⁺]. If this were true, then it would be difficult to reconcile with our observation that cPLA₂ activity appears to be constitutively associated with the membrane and is bound to phosphatidylinositol 4,5-biphosphate (32, 33). We do not question the validity of the widely reported [Ca²⁺]-dependent activation of some fraction of the type IV cPLA₂ that is known to be associated with the [Ca²⁺]-dependent translocation of the enzyme to the membrane. Our data suggest, however, that activation of cPLA₂ associated with BK channels may specifically involve a pool of cPLA₂ that is already located at or near its substrate in the membrane and is bound to phosphatidylinositol 4,5-biphosphate or other phospholipids.

In summary, our data point to cPLA₂-α as one target protein for phosphorylation that is intimately associated with the BK channel protein. Although we cannot unequivocally rule out the BK channel protein itself as an additional target for phosphorylation, the data that show a decrease in BK channel activity in the presence of ATP and the absence of cPLA₂ suggest that direct phosphorylation of the channel protein would serve to decrease rather than increase activity.

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