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Biochemical Pharmacology 66 (2003) 1363–1369
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Basic fibroblast growth factor (bFGF) regulation of the plasma membrane calcium ATPase (PMCA) as part of an anti-apoptotic mechanism of action

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Received 20 January 2003; accepted 4 April 2003

Abstract

Basic fibroblast growth factor (bFGF) preserves the viability of at least 13 different cells, including epithelial, endothelial, smooth muscle and neuronal cells. In spite of this profound and rather universal effect on cell viability, detailed studies regarding the mechanism of bFGF's action have not been conducted. Rather, most studies have simply shown that bFGF inhibits cells from undergoing programmed cell death (i.e. apoptosis). The most mechanistic studies to date have been conducted on either neurons or ovarian (granulosa) cells. These studies have shown that bFGF prevents apoptosis through both genomic and acute actions. Basic FGF's acute actions involved the maintenance of normal levels of intracellular free calcium levels ($[Ca^{2+}]_i$). In granulosa cells, bFGF maintained $[Ca^{2+}]_i$ through a protein kinase C_{delta} (PKCδ)-dependent mechanism. Further, bFGF-activated PKCδ maintained $[Ca^{2+}]_i$ by stimulating calcium efflux. The ability of bFGF to stimulate calcium efflux involved the plasma membrane calcium ATPase (PMCA). Interestingly, bFGF-activated PKCδ appeared to regulate PMCA activity in part by promoting its membrane localization.

Keywords: Basic fibroblast growth factor; Apoptosis; Ovary; PKCδ; Plasma membrane calcium ATPase; Granulosa cell

1. Introduction

Programmed cell death or apoptosis is a physiological cell ''suicide'' mechanism found in almost all multi-cellular organisms. Activation of this ''suicide'' cascade functions to eliminate cells as part of the normal embryonic development of organs. In adult organs, regulating the rate of apoptosis along with cell proliferation and differentiation controls tissue homeostasis [1–3].

Because apoptosis is a central component of the mechanism that controls tissue homeostasis, it has been suggested that all differentiated cells are programmed to undergo apoptosis. Further, it appears that apoptotic death is mediated through a conserved ''suicide'' cascade. The ''suicide'' cascade can be activated by two general pathways. The first pathway involves the activation of death receptors as typified by Fas/FasL system. The other is induced by various stresses such as when the levels of growth and/or survival factors become limiting. In the latter case, $[Ca^{2+}]_i$ levels often increase and remain elevated at least for several minutes. This prolonged increase in $[Ca^{2+}]_i$ is an essential component of the ''suicide'' cascade influencing both the execution phase of apoptosis by activating Ca^{2+}-dependent proteolytic enzymes and the initiation phase of apoptosis [4]. Briefly, most apoptotic stimuli deplete calcium levels within the endoplasmic reticulum [4]. This in turn increases $[Ca^{2+}]_i$. If $[Ca^{2+}]_i$ levels are not returned to a normal range within minutes, then the calcium concentration within the mitochondria increases. The increase intramitochondrial Ca^{2+} concentration is a key factor in determining the mode and amount of cytochrome *c* that is released [5]. The release of cytochrome *c* into the cytoplasm ultimately activates the caspase cascade, which commits the cell to die [6].

To prevent the activation of this conserved ''suicide'' cascade, most cells must be continuously exposed to survival factors. Basic FGF is one of the most common

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Abbreviations: bFGF, basic fibroblast growth factor; PKC, protein kinase C; $[Ca^{2+}]_i$, intracellular free calcium; PMCA, plasma membrane calcium ATPase; SIGCs, spontaneously immortalized granulosa cells.

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doi:10.1016/S0006-2952(03)00486-6

survival factors, maintaining the viability of several different cell types (see Table 1).

Basic FGF and its receptors are expressed in a specific spatial and temporal pattern in many different cells and tissues [7,8]. In addition, genetic approaches have shown that bFGF plays important roles in various developmental processes, such as angiogenesis, secretion and wound healing [7,8]. Although it is involved in all of these

Table 1
The effect of basic fibroblast growth factor on the cell survival

Cell type		Specific responses to basic fibroblast growth factor
Neuron-like cells	Human teratocarcinoma cells	These cells differentiate into neuronal-like cells. Basic FGF prevents apoptosis *in vitro* [34].
	Human glioma cells (U251MG)	Overexpression of a kinase defective mutant of the type I FGF receptor (i.e. a dominant negative FGF receptor) inhibits growth and induces apoptosis of U251MG cells [35].
	Chick retinal neurons	Basic FGF acts to maintain, bcl-x(L) and bcl-2 levels and prevents apoptosis [36].
	Hippocampal neurons	Basic FGF blocks glutamate-induced cell death *in vitro* and *in vivo*. It does so by up-regulating glial-cell-line-derived neurotrophic factor [37].
	Cerebellar granule cells	Basic FGF provides neuroprotection against ethanol-induced cell death [38].
	P19 embryonal carcinoma cells	Basic FGF activates phosphatidyl-inositol-3-kinase (PI3K) and stimulates the phosphorylation of Bad, which protects against activation of caspase-3 and apoptosis [39].
	Cortical neurons	Basic FGF inhibited Ca^{2+} ionophore-induced neurotoxicity, which is due to an increase in $[Ca^{2+}]_i$ [40].
	Hippocampal neurons	Basic FGF protects against nitric oxide-induced neuronal cell death [41].
	Cortical neurons	Basic FGF prevented neuronal death by maintaining Bcl-2 protein levels and attenuating caspase-3-like activity [42].
	Oligodendroglial cells	Basic FGF prevents apoptosis *in vitro* [43].
	PC12 neuronal cells	Basic FGF's anti-apoptotic action is mediated through both PKCδ and Ras/MAP kinase pathways [44].
Cancer cells	Human breast cancer and ovarian cancer cells	Basic FGF sensitizes MCF7 human breast cancer cells and A2780 human ovarian cancer cells to cisplatin [45].
	Small cell lung cancer cells	Basic FGF up-regulates Bcl-X(L) and Bcl-2 in a MEK-dependent fashion within 4 hr of treatment [46].
Endothelial cells	Endothelial cells	Basic bFGF protects against lethal effects of radiation [47].
	Endothelial cells	The anti-apoptotic effect of bFGF is dependent on tyrosine phosphorylation but not on activation of the MAP kinase pathway. Basic FGF inhibits apoptosis by both Bcl-2-dependent and independent mechanisms [48].
Muscle cells	Primary cardiac myocytes	Basic FGF is a protective factor against myocardial cell apoptosis and that this protection requires the MEK-1–ERK pathway [49].
	Vascular smooth muscle cells	Expression of dominant negative FGF receptor provoked apoptosis [50].
Ovarian cells	Ovarian surface epithelial cells	Basic FGF prevents apoptosis *in vitro* [10].
	Ovarian surface epithelial cells	Basic FGF blocks apoptosis induced by hepatocyte growth factor. It does so by maintaining low basal $[Ca^{2+}]_i$ levels [48].
	Granulosa cells	Basic FGF prevents apoptosis of granulosa cells cultured within ovarian follicles [9].
	Granulosa cells	Basic FGF maintains the viability of these cells in tissue culture [10].
	Granulosa cells	Extracellular matrix (EM) binds bFGF, which could account for EM's anti-apoptotic action [11].
	Immortalized human ovarian granulosa cells	Basic fibroblast growth factor (bFGF) effectively blocks the p53/cAMP-induced apoptosis [17].
	Spontaneously immortalized granulosa cells	Basic FGF prevents apoptosis by regulating intracellular free calcium levels through a PKCδ-dependent pathway [51].
Other	Gastric mucosal cells	*In vivo* studies indicate that bFGF blocks apoptotic caspase signaling cascade [52].
	Cytotrophoblasts and syncytiotrophoblasts	Basic FGF provides partial protection against TNF-alpha/IFN-gamma induced apoptosis [53].
	Human lens epithelial cell	Basic FGF maintains the viability of these cells in tissue culture [54].
	L929 mouse fibroblasts	Basic FGF protects cells from apoptosis by activating the Raf/MEK/MAPK pathway [55].
	NIH 3T3 cells	Basic FGF up-regulates Bcl-2 and delays apoptosis [56].
	Melanocytes	Direct anti-apoptotic effect of bFGF *in vitro* [57].
	Retinal pigmented epithelial cells	Basic FGF prevents apoptosis. The mechanism involves ERK-dependent synthesis of BCL-x [58].

processes, bFGF's precise mechanism of action has not always been clearly defined. Basic FGF's anti-apoptotic action, along with its ability to influence cell migration, proliferation and differential gene expression, is likely to be important factor in controlling each of these physiological processes. Therefore in this paper, studies will be presented that are directed toward elucidating the mechanism by which bFGF promotes ovarian granulosa cell survival. It is hope that these studies will provide insights that may be applicable to bFGF's actions in other cell types.

2. Basic fibroblast growth factor and ovarian cell survival

Several studies from various labs have shown that bFGF maintains the viability of cultured primary granulosa cells [9–12] and spontaneously immortalized granulosa cells (SIGCs) [10,13,14]. Previous studies have also shown that granulosa cells within healthy ovarian follicles express high affinity FGF receptors [15]. Further, bFGF is present within healthy ovarian follicles throughout the course of their development [15]. These findings imply that bFGF plays an important role in maintaining granulosa cell viability during follicular development [15]. Recent *in vivo* studies have also demonstrated that a mutation in the FGF receptor 3 results in granulosa cell apoptosis [16]. Finally, overexpression of bFGF reduces granulosa cell apoptosis with only 25% of the follicles possessing apoptotic granulosa cells in these mice compared to 58% for the wild-type controls.[1] Taken together, these studies provide compelling evidence that bFGF is a physiologically relevant regulator of granulosa cell viability.

3. Basic FGF and the regulation of intracellular calcium

In spite of its well-described role as a granulosa cell survival factor, little is known about the cellular and molecular mechanism through which bFGF prevents apoptosis. The activation of the FGF receptors by bFGF induces receptor dimerizaiton and tyrosine kinase activity [8]. Moreover, studies have shown that genistein, a tyrosine kinase inhibitor, blocks bFGF's ability to prevent granulosa cell apoptosis [9]. While this is consistent with bFGF's known mechanism of action, studies have not been conducted to elucidate the anti-apoptotic signaling events downstream of the tyrosine phosphorylation of the FGF receptor. It is likely that the downstream signaling events involve both acute and genomic actions [13,17–19]. Although the genomic actions are important and merit further study, the acute actions are critical since bFGF deprivation for just 30 min commits granulosa cells to undergo apoptosis [13]. Thus, the subsequent discussion will focus on the acute actions that mediate bFGF's anti-apoptotic effect.

The acute actions of bFGF appear to be related to changes in $[Ca^{2+}]_i$. This is based on the observations that (1) an increase in $[Ca^{2+}]_i$ occurs prior to either granulosa cells or SIGCs undergoing apoptosis, (2) this increase in $[Ca^{2+}]_i$ is sufficient to induce apoptosis since the intracellular calcium chelator, BAPTA, prevents apoptosis even in the absence of bFGF, (3) thapsigargin-induced increase in $[Ca^{2+}]_i$ promotes apoptosis in the presence of bFGF, and (4) bFGF prevents an increase in $[Ca^{2+}]_i$ in both granulosa cells and SIGCs [13,14,20].

Interestingly, the anti-apoptotic effect of bFGF is mimicked by an activator of PKC (i.e. a phorbol ester) and attenuated by PKC inhibitors, including the PKCδ inhibitor, rottlerin [13,14]. Further, PKC inhibitors abrogate the ability of bFGF to maintain normal basal $[Ca^{2+}]_i$. Moreover, PKCδ is the only PKC isotype that is detected in granulosa cells and SIGCs that can be activated by phorbol ester [13,14]. Moreover, treatment with either a PKCδ antibody or a specific PKCδ substrate peptide increases $[Ca^{2+}]_i$ compared to the respective control (i.e. IgG or PKCε substrate peptide) [14]. Finally, bFGF has been shown to activate PKCδ [14]. Taken together, these findings indicate that PKCδ accounts for bFGF's ability to regulate $[Ca^{2+}]_i$ and granulosa cell viability.

How then might bFGF-activated PKCδ regulate normal basal $[Ca^{2+}]_i$ levels? There are at least three possible sites of actions [21,22]. PKCδ could regulate (1) the release and/or uptake of calcium from its intracellular stores, (2) the entry of calcium from the extracellular fluid, and (3) the rate at which calcium is removed from the cell. While bFGF-activated PKCδ could affect any or all of these sites of action, recent studies have shown that bFGF stimulates calcium efflux. This action is also attenuated by the PKCδ inhibitor, rottlerin [14]. Although the mechanism that regulates calcium efflux is complex, one enzyme that plays a major role in promoting calcium efflux in most cells is the PMCA. Further, inhibitors of PMCA block the ability of bFGF to stimulate calcium efflux, maintain $[Ca^{2+}]_i$ and prevent granulosa cell apoptosis [14]. Based on these observations, bFGF's ability to promote calcium efflux appears to be mediated at least in part by PMCA. That overexpression of PMCA reduces calcium-mediated cytotoxicity in PC12 cells further supports a role for PMCA in cell survival [23]. Thus, the regulation of PMCA activity is likely to be an important part of the mechanism through which bFGF regulates $[Ca^{2+}]_i$ and cell viability.

4. PKCδ, plasma membrane calcium ATPase and ovarian function

There are four known isoforms of PMCA with each form having three to four splice variants. Through the use of a

[1] Peluso JJ, unpublished observations.

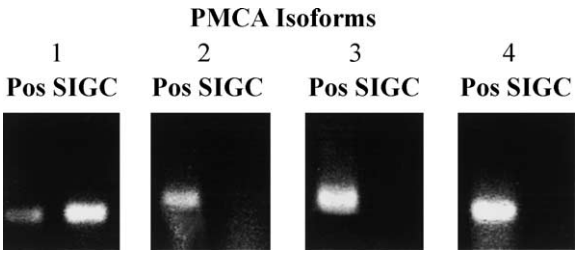

Fig. 1. RT–PCR analysis of the expression of PMCA isoforms in SIGCs. An RT–PCR product generated from mRNA isolated from rat brain is shown as a positive control (Pos) for each PMCA isoform [24]. For this procedure the following primer pairs were used. The size of the PCR product is shown in the parentheses. PMCA 1: sense, CCA TGC CGA ACG GGA GTT GC; antisense, CG GAG GGC TGG AGT TGC GTT (bp 259); PMCA 2: sense, CAC CAT CCC TAC CAG CAG GC; antisense, CA GGT CGG TGT CAT CGA TGA (bp 292); PMCA 3: sense, GTC ATT GCC ACT ATC CC; antisense, AG CTA CGG AAT GCT TTC ACC (bp 346); PMCA 4: sense, ATG CCG AGA TGG AGC TTC GC; antisense, CA GCA TCC GAC AGG CGC TTG (bp 259). For each isoform the sample was denatured at 94° for 30 s, annealed at 50° for 30 s and elongation at 72° for 60 s. After 30 cycles, PCR products were resolved by electrophoresis on a 1.5% agrose gel and stained with ethidium bromide.

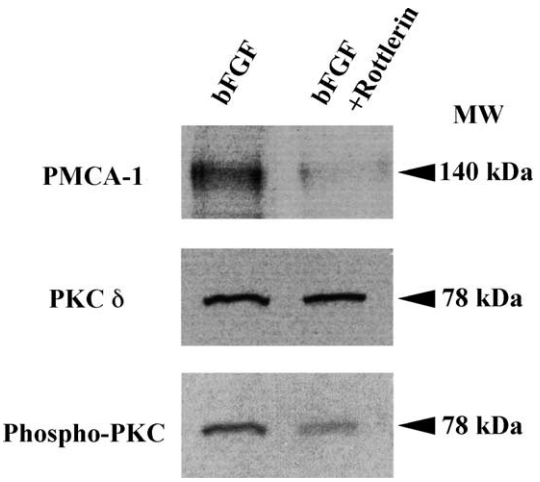

Fig. 3. The effect of bFGF and rottlerin on the membrane levels of PMCA, PKCδ and phosphoPKC. Note that rottlerin does not decrease the level of PKCδ but does reduce PMCA levels. Also PKCδ activity (i.e. level of phosphoPKC) is directly correlated with the amount of PMCA that is localized to the membrane. The treatment and protocol involving the preparation of the membrane fractions were conducted as described by Peluso *et al.* [14]. This experiment was replicated six times with essentially the same result.

RT–PCR protocol, which identifies each of these PMCA forms and their splice variants [24], PMCA-1b was shown to be the only form of PMCA that is expressed by SIGCs (Fig. 1). This has been confirmed by both Western blot analysis and immunocytochemistry (Fig. 2).

PMCA can be phosphorylated PKC (for review, see [25]). However, compared to other regulators, such as calmodulin, PKC has a relatively minor effect of PMCA activity when assessed on purified PMCA [25]. Interestingly, in intact granulosa cells, PKCδ appears to promote the membrane localization of PMCA. This is based on the observation that rottlerin, a PKCδ inhibitor, reduces the amount of PMCA within a crude membrane fraction within 2.5 min (Fig. 3). The reduction in membrane levels of PMCA correlates with a reduction in phosphoPKC levels (i.e. PKCδ activity). Since PMCA must be at the plasma membrane to expel calcium, disrupting the membrane localization of PMCA should have a profound effect on bFGF's ability to regulate calcium homeostasis and cell viability.

In addition, it appears that in healthy SIGCs some of the membrane-associated PMCA is internalized. This is based on the observation that PMCA, which was biotinylated along with the other surface membrane proteins at the start of culture, is detected within the cytoplasm within 1 hr of culture (Fig. 4). This indicates that in healthy SIGCs some

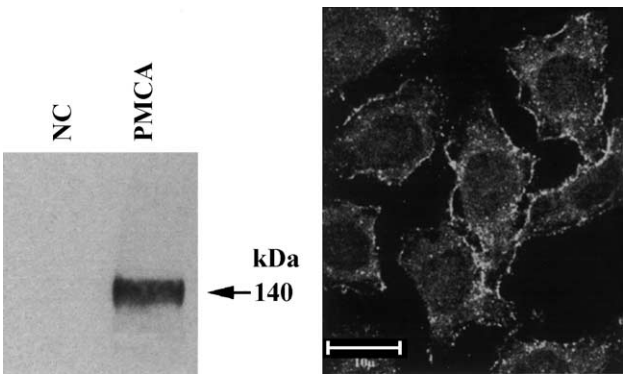

Fig. 2. The expression and localization of PMCA in SIGCs as revealed by Western blot (left panel) and confocal microscopy (right panel). In the left panel, NC indicates a negative control. As seen in the confocal image, PMCA is localized to select areas of the plasma membrane and is also detected as punctate vesicles within the cytoplasm. Staining was not detected in the negative controls. The Western blots were conducted as previously described [14] using the primary PMCA antibody (MA3-914, Affinity Bioreagents, Inc.) at a 1:500 dilution.

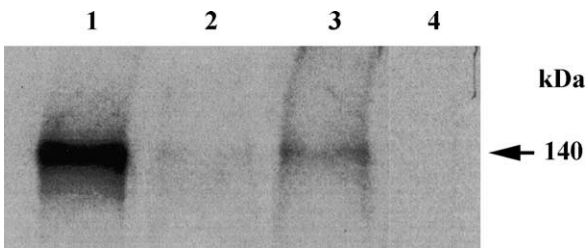

Fig. 4. Internalization of surface-biotinylated PMCA. SIGCs were biotinylated and then affinity purified with streptavidin. In lane 1 affinity purified proteins were then subjected to Western blot for the detection of PMCA. In lane 2 SIGCs were exposed to glutathione to remove the biotin from the surface proteins then treated as the lysate in lane 1. In lane 3, SIGCs were biotinylated, cultured for 1 hr at 37°, exposed to glutathione, affinity purified and processed for PMCA Western blot analysis. A negative control using the same lysate as in lane 1 is also shown lane 4. In this negative control the Western blot procedure was conduced in the absence of the primary antibody. This study was replicated three times with essentially identical results. This study was conducted following the protocol outlined by Le *et al.* [59].

PMCA is normally internalized. Whether bFGF-activated PKCδ regulates the rate at which PMCA is internalized remains to be determined.

It is likely then that bFGF-activated PKCδ phosphorylates proteins that are involved in maintaining the membrane localization of PMCA. The mechanism involved in maintaining PMCA and other ion pumps and exchangers is just beginning to be elucidated. It is known that the sodium/hydrogen exchanger regulatory factor-2 (NHERF-2) binds to the PDZ binding site of PMCA [26]. NHERF-2 also can associate with either ezrin, radixin or moesin [27] which in turn bind to cortical actin cytoskeleton. Moreover, moesin [28] and NHERF-2[2] are expressed by SIGCs. In addition, PKCθ, a non-conventional PKC isoform like PKCδ, has been shown to phosphorylate and thereby activate moesin's ability to bind to cortical actin cytoskeleton [29]. Further, both PKCθ and PKCδ have the same specific substrate, which is 75% homologous to the phosphorylation site of moesin [30]. These observations that make it likely that one of the molecular targets of PKCδ is moesin. PKCδ-activation of moesin could link the PMCA–NHERF-2 complex to the actin cortical skeleton and be part of the mechanism by which PMCA is directed to the plasma membrane. While this hypothesis is interesting, more studies must be conducted to confirm it.

5. Conclusions and future research

The studies presented in this review provide the framework for the novel concept that bFGF-activated PKCδ controls $[Ca^{2+}]_i$ and subsequently cell viability by maintaining PMCA's membrane localization. It is proposed that when PKCδ is active (i.e. in the presence of bFGF or serum), PMCA is continuously internalized and then recycled to the plasma membrane. Conversely if PKCδ activity is reduced or inhibited, then it is predicted that PMCA would be rapidly internalized probably via an endocytotic mechanism but not returned to the plasma membrane. In support of this concept, insulin has been shown to translocate the sodium–potassium pump to the plasma membrane of muscle cells within minutes of exposure [31]. Further, the sodium–potassium pump has been shown to be endocytosed within 2.5 min of dopamine exposure [32]. Interestingly, the membrane localization of this pump is regulated by PKC in an isotype specific manner that is dependent on an intact actin cytoskeleton [30]. However, the present observations only provide the framework for the concept that the bFGF-activated PKCδ directs and maintains PMCA at the plasma membrane. Much more work must be done in order to confirm this putative regulatory mechanism.

Finally, it is important to appreciate that bFGF not only signals through a PKC-dependent mechanisms but also through numerous other kinase cascades including phospholipase Cγ, Src kinases, Ras-MAP kinase pathway, phosphatidylinositol 3′ kinase and focal adhesion kinase [33]. Further, SIGCs and granulosa cells express the sodium–calcium exchanger (NCX) and the sodium–potassium ATPase pump (NKA).[3] Nothing is known about bFGF's role in regulating the ability of NCX/NKA to work together to promote calcium efflux and regulate $[Ca^{2+}]_i$ levels [21]. Because of the complex multi-faceted signaling cascades that are regulated by bFGF, it is possible that bFGF regulates calcium efflux by controlling both the PMCA and NCX/NKA pathways. If so, then PMCA and NCX/NKA pathways may exist and function as either redundant or complimentary mechanisms through which bFGF controls $[Ca^{2+}]_i$. This in fact may be likely, since the regulation of $[Ca^{2+}]_i$ is critical to granulosa cell survival.

Acknowledgments

The author would like to thank Anna Pappalardo, Gus Fernandez and Kelly Lynch for their assistance in conducting the experiments outlined in this review. The author would also like to acknowledge the thoughtful insights provided by Bruce White of the Department of Physiology, University of Connecticut Health Center. This work was supported in part by NIH grant HD33467.

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