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CHARACTERIZATION OF MEMBRANE-ASSOCIATED SUBSTRATES OF Ca^{2+}-DEPENDENT KINASES IN ASTROCYTES

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1. ABSTRACT

Membrane-associated kinase substrates are likely transducers of extracellular signals elicited by neuroimmunomodulators and other signaling molecules. Whereas specific signal transduction pathways in astrocytes are being defined, the global view is lacking. We, therefore, characterized membrane-associated substrates of Ca^{2+}-dependent kinases in primary astrocytes using 2-dimensional gel electrophoresis. Ten proteins were phosphorylated *in vitro* and characterized with respect to their relative molecular mass (in the range 10 kDa – 100 kDa), isoelectric point (range 4.2 – 9.0) and four conditions of phosphorylation. They varied broadly in their requirements for phosphorylation displaying distinct kinase preferences. Eight phosphoproteins were substrates of protease kinase C. Judging by abundance and intensity of phosphorylation, the principal PKC substrates were three acidic proteins associated with the plasma membrane. These results suggest that a relatively small number of membrane-associated proteins serve as transducers of signals mediated by Ca^{2+}-dependent kinases and most of them are PKC substrates in astrocytes.

2. INTRODUCTION

A large body of recent data indicates that several cytokines, such as IL-1β and TGF-β1, as well as other neuromodulators, such as FGF-2 and endothelin, act on astrocytes via protein phosphorylation (1-5). Protein phosphorylation is a principal means of regulating cellular processes. Thus, it is no surprise that protein kinases constitute a very large family of enzymes encoded by more than 2000 genes in the human genome (6). The substrate specificity of brain protein kinases is broad, as demonstrated by a large number of neural proteins which have been reported to be phosphorylated both *in vitro* and *in vivo* (7-10). New methodologies being developed for the global analysis of protein phosphorylation testify to the importance of this approach to understanding cellular processes. For example, a combination of immobilized metal-affinity chromatography followed by nanoflow HPLC/electrospray ionization mass spectrometry revealed hundreds of phosphorylation sites in a whole-cell lysate of yeast (6). Dynamics of protein phosphorylation in single living cells can now be visualized with genetically encoded fluorescent indicator (11). This is a fusion protein consisting of a substrate domain for a protein kinase of interest linked to a "phosphorylation recognition domain" and sandwiched between two green fluorescent protein mutants of different color. A phosphorylation event provokes intramolecular binding of the recognition and substrate domains and fluorescence resonance energy transfer that is visualized (11).

Protein phosphorylation in astrocytes has been increasingly recognized as an important pathway for regulation of astrocyte function. Characterization of astrocytic proteins that are phosphorylated by protein kinases contributes to understanding the ability of these cells to interact with their environment and mediate neuro-immune interactions. This is particularly important in astrocytes as they serve a homeostatic role in the brain and modify neuronal function in a variety of ways. Relative molecular masses and isoelectric points of some kinase substrates in astrocytes (e.g. glial fibrillary acidic protein (GFAP) and vimentin) have been reported (12). However, no global survey of membrane-associated substrates of Ca^{2+}-dependent kinases in astrocytes has been reported. These proteins are important because they are likely near-

Table 1. Characterization of Membrane Phosphoproteins in Astrocytes

	Protein Species (number) [1]									
Molecular parameters	1	2	3	4	5	6	7	8	9	10
• Relative molecular mass (kDa)	53	80	43	39	69	89	80	46	54	52
• Isoelectric point (pI)	5.5	3.9	4.1	7.8	7.9	7.8	7.4	5.6	5.6	7.7
Phosphorylation conditions[2]										
• Membrane proteins (MP) (42 μg)	36.1	5.30	17.4	9.61	0	8.40	0	12.2	10.9	0
• MP plus Ca^{2+} (100 μM)	44.5	20.0	2.80	4.50	0	3.80	0	6.30	3.60	14.5
• MP plus Ca^{2+}, phosphatidyl-L-serine • PS (P; 40 μg/ml), diolein (D;1 μg/ml),	40.0	21.3	16.5	7.50	0	6.80	7.90	0	0	0
• MP plus Ca^{2+}, calmodulin (100 μg/ml)	65.3	0	0	11.9	4.80	6.90	0	7.30	3.80	0

[1]The numbers refer to proteins as designated in Figure 1.[2] The phosphorylation was by endogenous kinases in the presence of added [^{32}P]ATP and other compounds, as stated. (For details, see the Methods). It was quantitated by measuring optical density (OD) of spots on autoradiograms of 2-dimensional gels. The numbers are OD as a percent of total, for each condition. The results are representative of three independent experiments.

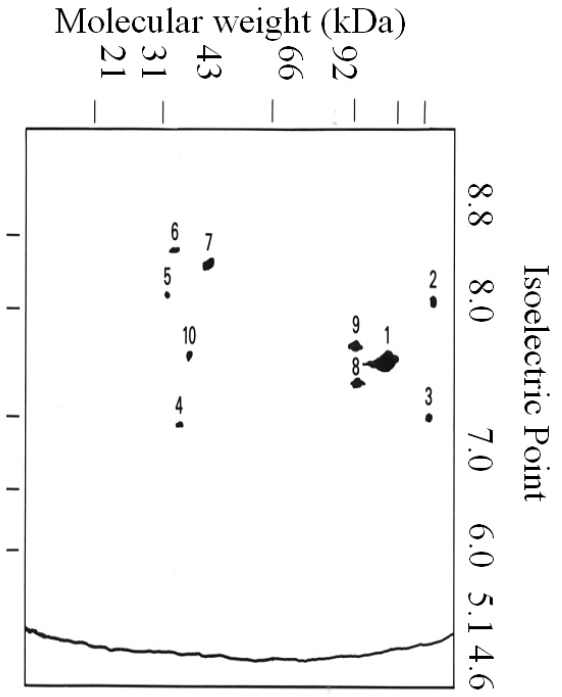

Figure 1. Silver stained gel showing membrane-associated proteins isolated from rat astrocytes grown in primary culture. Membrane proteins were resolved by 2-dimensional electrophoresis and visualized by silver staining and autoradiography. The data are representative of three independent experiments.

proximal transducers of extracellular signals and potential drug targets. To begin to bridge this gap we used *in vitro* phosphorylation and 2-dimensional gel electrophoresis to characterize 10 membrane-associated substrates of Ca^{2+}-dependent kinases in astrocytes. In a companion paper we identify one of three principal substrates of protease kinase C (PKC) (13).

3. MATERIALS AND METHODS

3.1. Cell Culture

Astrocytes were isolated by mechanical dissociation from neonatal rat cortex tissue and cultured as previously described (14,15). Briefly, cells were plated in 75 cm^2 flasks and grown in DMEM/F12 (1:1 v/v) supplemented with 10% fetal calf serum for 7-10 days until confluent. The cultures contained 95-98% astrocytes as judged by staining with an antibody to GFAP. Astrocytes and other cell types were identified by immunolabeling as previously described (15,16). No cells were passaged before use.

3.2. Isolation of membranes

Cell fractionation and isolation of membranes was performed as previously described (16,17). Briefly, cells in a monolayer were rinsed with 5 mM tris-HCl buffer pH 8 containing protease inhibitors, 0.5 mM EDTA and 17 μM PMSF (phenylmethylsulphonyl fluoride). Cells were scraped with a "rubber policeman" into this solution containing the following additional protease inhibitors: aprotonin, antipain chymostatin, and leupeptin; all at 5 μg/ml. All protease inhibitors were present throughout subsequent manipulations. Cells were homogenized in Wheaton homogenizer without disintegration of nuclei as observed with a microscope. Cellular homogenate was centrifuged at 890 x g for 5 min and the pellet, containing nuclei and intact cells, discarded. The supernatant was centrifuged at 30,000 x g for 20 min yielding "cytosolic" (supernatant) and "microsomal" (pellet) fractions. The microsomal fraction, containing plasma and organelle membranes, was the source of "membrane-associated" proteins (e.g. Figure 1).

3.3. Protein Phosphorylation

Determination of the protein species which serve as kinase substrates was performed in vitro with either endogenous kinases (i.e. present in extracts of astrocytes) or partially-purified PKC as previously described (16,17). (Brain cortices from Sprague-Dawley rats were the source of exogenous PKC.) Briefly, proteins from astrocytes or one-day-old neonate rat cortex were added to a reaction mixture containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1.0 mM EGTA, 30 μM [32P]ATP (specific activity 6,600 cpm/pmole), and one, or a combination of more than one (see Table 1) of the following reagents: 1.1 mM CaCl2 (approximate final concentration 100 μM CaCl2) phosphatidyl L-serine (40 μg/ml), diolein (1 μg/ml), and calmodulin (0.1 μg/ml). The reaction lasted 1 min and was stopped by freezing.

3.4. Electrophoresis

Proteins were resolved by 2-dimensional polyacrylamide gel electrophoresis (PAGE) as previously

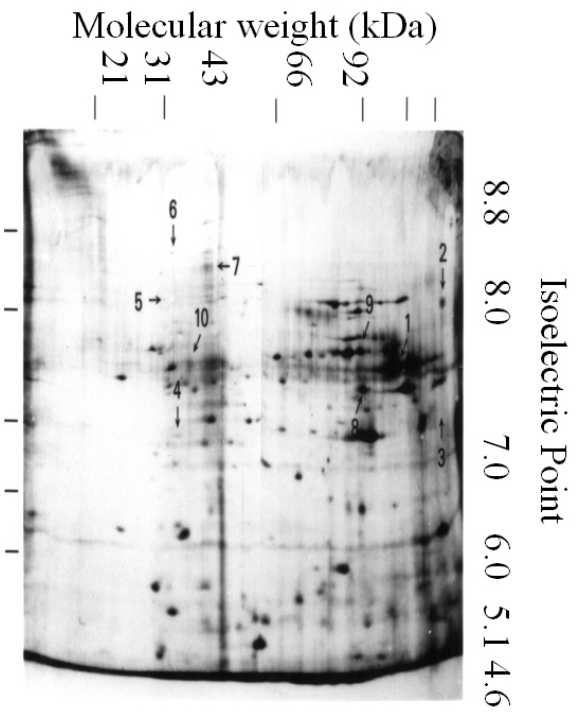

Figure 2. Ten astrocytic membrane proteins were phosphorylated *in vitro* by Ca^{2+}-dependent kinases. Composite drawing showing spots, revealed by autoradiography of 2-dimensional gels, of all membrane-associated proteins phosphorylated by endogenous Ca^{2+}-dependent kinases *in vitro*. Note that some phosphoproteins (e.g. pp3) were not detected by silver staining but were detected after phosphorylation and autoradiography.

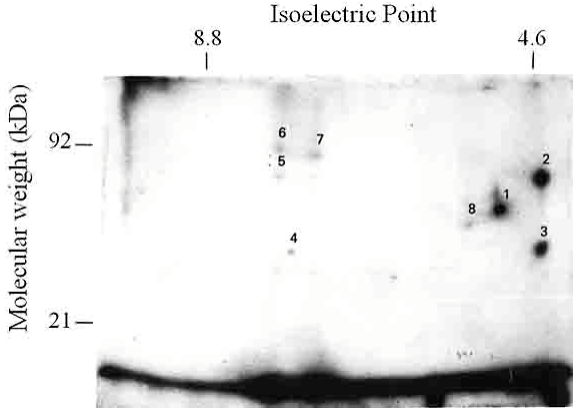

Figure 3. PKC substrates in membranes from cultured astrocytes. Membrane proteins (40 µg) were phosphorylated in vitro by partially purified, exogenous, rat brain PKC in the presence of [32P]ATP, Ca2+, phosphatidyl-L-serine and diolein. After phosphorylation, they were resolved by 2-dimensional electrophoresis and visualized by autoradiography. Among eight proteins that were detected, three acidic ones were most intensely phosphorylated as judged by the optical density of the spots. The data are representative of three independent experiments.

described (16,17). After electrophoresis, proteins were either fixed, stained and/or autoradiographed as previously described (16,17). The spots on 2-dimensional autoradiograms were quantified by scanning densitometry with an ultrascan XL laser densitometer (LKB).

3.5. Materials

The seven protease inhibitors were purchased from Sigma. Phosphatidyl serine (PS) diolein (D), calmodulin (CAM) and myelin basic protein also were from Sigma (St. Louis, MO). Leupeptin was from Boehringer-Mannheim (Indianapolis, IN).

4. RESULTS

4.1. Abundance of Membrane-Associated Ca^{2+}-Dependent Kinase Substrates in Rat Brain Astrocytes

Two-dimensional gel electrophoresis resolved proteins from rat astrocytes grown in a primary culture, in the molecular mass range between 10 kDa and 100 kDa and pI range between 4.2 and 9.0 (Figure 1). Endogenous, membrane-associated kinases phosphorylated approximately 10 proteins under four conditions tested *in vitro* (Table 1, Figure 2). We designated these phosphoproteins (pp) by number from 1-10 and depicted them in a composite drawing shown in Figure 2. Thus, about 3 % of proteins in the Mr and pI ranges tested, are potential signal transducers. Judging by the intensity of protein staining by silver, phosphoprotein 1 (pp1) was the most and pp3 the least abundant (Figure 1). When cytosolic proteins were phosphorylated by protein kinases pp1, pp2, pp3, and pp8 were detected in that subcellular fraction (data not shown). This suggests that four phosphoproteins are partitioned between cytosol and membranes whereas six are integral membrane proteins.

4.2. Specificity of phosphorylation

Seven proteins were phosphorylated in basal condition i.e. in the absence of added Ca^{2+} and other compounds (Table 1). Three proteins, pp1, pp4 and pp6 were phopshorylated in all conditions tested. In contrast, another three were phosphorylated in only one condition, each. Pp10 was phosphorylated only in the presence of added Ca^{2+} but not additional compounds; pp5 required Ca^{2+} and calmodulin, and pp7 required all three additives, Ca^{2+}, phosphatidyl serine and diolein (Table 1). When compared to the basal condition, phosphatidyl serine and/or diolein inhibited phosphorylation of pp8 and pp9 whereas calmodulin inhibited phosphorylation of pp2 and pp3. Pp4 and pp6 were substrates for both, PKC and Ca^{2+}/calmodulin kinases. These results indicate that membrane-associated phosphoproteins in astrocytes vary broadly in their requirements for phosphorylation by endogenous Ca^{2+}-dependent kinases. This was not due to proteolysis because the assays were done in the presence of seven protease inhibitors. However, dephosphorylation may have contributed to these results because we did not use phosphatase inhibitors. The data indicate that the phosphorylation *in vitro* was specific in that some proteins displayed kinase preference.

4.3. Intensity of phosphorylation

To estimate the extent of phosphorylation of one protein relative to the others, we measured optical density

(OD) of each spot in an autoradiogram and expressed it as a fraction of total OD (OD of all spots in one autoradiogram added together) (Table 1). This approximate measure does not take into account the abundance of a protein. Pp1 was most intensely phosphorylated under all the conditions tested. Pp1 was also the most abundant protein as judged by the intensity of staining with silver (Figure 1). Pp2, pp6 and pp9 were the least phosphorylated judging by the OD of their spots. These results suggest that the intensity of phosphorylation in a given condition, varied from protein to protein.

4.4. Putative PKC substrates

Conventional PKC activity is optimal in the presence of Ca^{2+}, phosphadidyl serine and diolein. In this condition, six proteins were phosphorylated by endogenous, membrane associated kinases: 1, 2, 3, 4, 6, and 7 (Table 1). However, when we added to the reaction mix PKC partially purified from rat brain, two additional proteins corresponding in Mr and pI to pp5 and pp8, were visualized (Figure 3). Note that the pI of PKC prepared the way we did, is 5.6 (18). Although PKC autophosphorylates, it is not visible in the autoradiogram shown in Figure 3, because it was at a very low concentration. These results indicate that 8 of 10 membrane-associated phosphoproteins in astrocytes are PKC substrates. Judging by the OD of the spots, the principal PKC substrates were the acidic proteins, pp1, pp2 and pp3.

5. DISCUSSION

The data presented here show that astrocytes from rat brain and grown in a primary culture contain approximately 10 membrane-associated proteins (in the Mr range 10 kDa - 100 kDa and pI range 4.2 - 9.0), that were phosphorylated *in vitro* by endogenous membrane-associated Ca^{2+}-dependent kinases. They differed in kinase preference, partitioning between cytosol and membranes and regulation by CAM. Thus, a finite, relatively small number of membrane-associated proteins in astrocytes are substrates of Ca^{2+}-dependent kinases. Three acidic proteins are likely major substrates of PKC as judged by abundance and intensity of phosphorylation. Pp1 was phosphorylated under all tested conditions and was the most abundant phosphoprotein judging by intensity of staining with silver. High abundance, Mr, pI, phosphorylation by Ca^{2+}- (Table 1) and cAMP-dependent (PKA, data not shown) kinases and partitioning between cytosol and membrane suggest that pp1 is similar, if not identical, to β-tubulin (19-21). We have previously identified pp2 as GAP-43 (16,17) and we now identified pp3 as MARCKS (13). Although the precise functions of the latter two proteins are still being worked out, they are involved in actin dynamics (22). Thus, principal, membrane-associated PKC substrates in astrocytes are structural, rather than regulatory, proteins also present in neurons. This suggests that PKC-mediated transduction mechanisms in astrocytes and neurons are, at least in part, similar to each other.

Membrane-associated phosphoproteins of astrocytes varied widely in their requirements for phosphorylation by endogenous Ca^{2+}-dependent kinases. Our assay measured steady-state phosphorylation and did not rule out a contribution of phosphatases. However, dephosphorylation, if any, was minimal because the assay lasted only one min. Phosphoproteins 1, 4 and 6 were phosphorylated under all tested conditions indicating an ability to serve as substrates for multiple kinases. Two other astrocytic phosphoproteins, GFAP and vimentin, are known to serve as substrates for both PKC and PKA (12). Thus, at least five proteins in astrocytes can mediate signals transduced by multiple kinases. In contrast, pp5, pp7 and pp10 are likely substrates for only one type of Ca^{2+}-dependent kinases, each. CAM stimulated (pp1, pp4, pp5, pp6, pp8 and pp9) and inhibited (pp2 and pp3) phosphorylation. Previous reports showed that phosphorylation of proteins with Mr and pI similar to pp2 and pp3 is inhibited by CAM (23,24). Thus, CAM regulates phosphorylation of some membrane-associated proteins in astrocytes.

Four phosphoproteins (pp1, pp2, pp3, and pp8) also were detected in cytosol (data not shown) indicating that they were partitioned between the cytoplasm and plasma membrane. Four proteins with molecular masses similar to the four described here were previously reported as cytosolic PKC substrates in astrocytes (25). Phosphorylation *in vitro* followed by 2-dimensional electrophoresis and autoradiography revealed a small number of membrane-associated proteins (Mr=10 – 100 kDa; pI=4.2 – 9.0) as substrates for Ca^{2+}-dependent kinases in astrocytes. This number needs to be corroborated in light of recent findings in yeast (6). These proteins are important because they are likely transducers of cytokine and other neuro-immunomodulatory signals and, therefore, their identification should proceed rapidly.

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