Characterization of calcium-dependent membrane binding proteins of brain cortex

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Proteins of M_r 68000, 34000 and 32000 were selectively extracted by EGTA from brain cortex. The three proteins that were extracted along with calmodulin were acidic, monomeric, and did not exhibit structural homology, as demonstrated by one-dimensional peptide mapping. The M_r -68000 protein was purified to homogeneity and had a Stokes radius of 3.54 nm and $s_{20,w}$ value of 5.1 S. Purified calmodulin, M_r -68000 protein and two proteins of M_r 34000 and M_r 32000, interacted with the brain particulate fraction, with half-maximal binding occurring at 3.5 μ M, 8.3 μ M and 150 μ M- Ca^{2+} respectively. Proteins were bound independently of each other and calmodulin. Pretreatment of the particulate fraction with trypsin prevented the Ca^{2+} -dependent binding of calmodulin; however, the binding of the M_r -68000 protein or the M_r -32000 and -34000 proteins was unaffected. The M_r -68000 protein of bovine brain did not cross-react immunologically with M_r -67000 calcimedins from chicken gizzard.

Ca^{2+} participates in a wide range of metabolic and motile events through its interaction with calmodulin and specific intracellular Ca^{2+} -binding proteins (Cheung, 1982; Campbell, 1983). Although calmodulin has been implicated in the mediation of secretory events in chromaffin cells (Baker & Knight, 1981) and at the nerve terminal (DeLorenzo, 1982), numerous proteins have been identified that may modulate Ca^{2+} -dependent interactions with the secretory vesicle membrane (Creutz *et al.*, 1983). Several chromaffin-granule-membrane proteins have been shown to bind calmodulin directly or to require calmodulin for their interaction with the granule membrane (Geisow & Burgoyne, 1982, 1983). A class of proteins designated 'calcimedins', which bind to hydrophobic matrixes in the presence of Ca^{2+} , have also been isolated from striated and smooth muscle (Moore & Dedman, 1982).

During EGTA extraction of calmodulin from the particulate fraction of brain cortex, additional proteins unique to the calmodulin extract were identified by SDS/polyacrylamide-gel electrophoresis in the present work. These proteins appear to be identical with those identified in brain and designated as 'calelectrins' by Südhof *et al.* (1984)

on the basis of immunological cross-reactivity with a protein (M_r 34000) termed 'calelectrin' isolated from *Torpedo marmorata*.

Materials and methods

Materials

Ultrapure reagents were obtained from Bethesda Research Laboratory, Bethesda, MD, U.S.A. Bovine brain was obtained within 1 h after slaughter and frozen until use. Peroxidase-coupled rabbit anti-sheep immunoglobulins were obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN, U.S.A.

Protein extraction

Calcium-dependent membrane binding proteins were purified from bovine brain by homogenization in a Waring blender for 1 min with 3 vol. of buffer A [25 mM-Tris/HCl (pH 7.5)/2.0 mM- MgCl_2 / 0.5 mM - dithiothreitol / 0.3 mM - phenyl-methanesulphonyl fluoride/200 μ M- CaCl_2] containing 150 mM-NaCl. The suspension was stirred for 30 min at 4°C and centrifuged at 15000g for 15 min. The pellet was resuspended and washed sequentially in 2 vol. of buffer A containing 300 mM-NaCl and twice in buffer A containing 150 mM-NaCl. The final washed pellet was resus-

Abbreviation used: SDS, sodium dodecyl sulphate.

pended in 1.5 vol. of buffer A containing 5 mM-EGTA. The supernatant of the EGTA extract was obtained by centrifugation for 40 min at 80000g.

Protein purification

The EGTA extract from 1.5 kg of bovine brain cortex was slowly made 55% satd. with solid $(\text{NH}_4)_2\text{SO}_4$ and maintained at pH 7.5 for 30 min. After centrifugation at 15000g for 30 min, the supernatant was adjusted to pH 4.3 with 0.5 M- H_2SO_4 , stirred for 35 min and centrifuged as above. The pellet was resuspended in 30–50 ml of 20 mM-Tris/HCl, pH 7.5, containing 0.5 mM-dithiothreitol. In some instances, chromatography on a fluphenazine-agarose column (prepared as described by Kakiuchi *et al.*, 1981; 1.5 cm $\times$ 20 cm) was employed at this stage to remove calmodulin. Routinely, the resuspended pellet was dialysed against 50 vol. of buffer B [20 mM-Tris/HCl (pH 7.5)/0.5 mM-dithiothreitol/0.1 mM-EGTA] for 12 h and applied to a 2.5 cm $\times$ 12 cm DEAE-Sephacel column. The column was washed with 100 ml of buffer B and the Ca^{2+} -dependent binding proteins were eluted as a single peak with a 500 ml linear gradient (0–0.6 M-NaCl) in buffer B. The pooled fractions based on SDS/polyacrylamide-gel electrophoresis were dialysed against 50 vol. of buffer B containing 2 mM- MgCl_2 and applied to a 1.5 cm $\times$ 18 cm column of Affi-Gel Blue-agarose. The column was washed with 60 ml of the dialysis buffer to elute unbound proteins of M_r 34000 and 32000. A 300 ml linear gradient (0–1.0 M-NaCl) in this same buffer was employed to elute the M_r -68000 protein. Both bound and unbound fractions were pooled, concentrated to 0.5–1.0 mg/ml and stored as a frozen suspension in 10% (v/v) glycerol containing 5 mM-Tris/HCl, pH 7.5, and 0.5 mM-dithiothreitol.

Calmodulins from chicken gizzard were prepared and purified as previously described (Moore & Dedman, 1982). The M_r -67000 calmodulin was prepared from the pooled fraction eluted from fluphenazine-Sephacel. The calmodulin fraction was dialysed against 10 mM-imidazole, pH 6.4, containing 1 mM-EDTA and separated on a DEAE-cellulose column (1.5 cm $\times$ 20 cm) equilibrated with dialysis buffer. After washing of the column to baseline A_{280} , the calmodulins were eluted with a 300 ml linear gradient of 0–120 mM-NaCl. The M_r -67000 calmodulin peak was pooled and further purified by EGTA elution from a phenyl-Sephacel column (1.5 cm $\times$ 20 cm) equilibrated with imidazole buffer containing 1 mM- CaCl_2 in place of EDTA. The M_r -67000 calmodulin was judged homogeneous by SDS/polyacrylamide-gel electrophoresis.

Polyclonal antibodies were prepared against the four calmodulins in sheep at Bethyl Laboratories,

Montgomery, TX, U.S.A. The sheep were injected at monthly intervals with 0.3–0.5 mg of antigen. For the isolation of specific polyclonal antibody, antiserum was applied to a M_r -67000-calmodulin-Sephacel affinity column (0.7 cm $\times$ 3.0 cm), and the column was washed with 0.2 M-glycine, pH 6.0. Antibody was eluted with 0.2 M-glycine, pH 2.7, and adjusted to pH 7.0 with Tris base. The affinity-purified antibody was dialysed extensively against 100 mM-sodium borate/25 mM-sodium pyrophosphate/76 mM-NaCl, pH 8.0. Calmodulin was purified from bovine brain as described by Kakiuchi *et al.* (1981) with modification (Ahluwalia *et al.*, 1984). All isolation procedures were performed at 4°C.

Polyacrylamide-gel electrophoresis

Electrophoresis was performed in the presence of SDS (Laemmli, 1970) on 9.5% or 6–15% (linear gradient) polyacrylamide slabs. Two-dimensional gel electrophoresis was performed by using ampholytes in the range pH 3.5–10 (Cabrall & Schatz, 1979). One-dimensional peptide mapping on polyacrylamide gels was as described by Cleveland *et al.* (1977) by using *Staphylococcus aureus* proteinase.

Binding studies of brain proteins

The final pellet after EGTA extraction of the brain particulate fraction was resuspended with a Teflon-pestle homogenizer in 3 vol. of buffer C [25 mM-Tris/HCl (pH 7.5)/2.0 mM- MgCl_2 /0.5 mM-dithiothreitol / 0.3 mM - phenylmethanesulphonyl fluoride] containing 300 μM -EGTA. After centrifugation at 105000g for 30 min, the pellet was washed sequentially with 3 vol. each of buffer C containing 50 μM - Ca^{2+} and without Ca^{2+} . The washed pellet was stored frozen without resuspension. Before assay, the particulate fraction was washed with 8 ml of buffer C and incubated in 0.4 ml of buffer C containing 100 mM-NaCl and either 0.5 mM- Ca^{2+} or 150 μM -EGTA. In certain experiments, free Ca^{2+} concentration was maintained with a 200 μM - Ca^{2+} -EGTA buffer (Bartfai, 1978). Purified calmodulin (19 μg), M_r -68000 protein (9.5 μg) and 19 μg of the purified fraction containing the M_r -34000 and -32000 proteins were added alone or in combination and incubated at 30°C for 20 min. The mixtures were then diluted to 4.6 ml with incubation buffer containing Ca^{2+} at fixed concentration or EGTA alone (control). After centrifugation as above, the pellet was extracted with 2.0 ml of buffer C containing 5 mM-EGTA for 20 min at 4°C. Extracts were centrifuged at 105000g for 1 h and the supernatant was concentrated by freeze-drying and applied to a SDS/polyacrylamide gradient gel. The gel was stained with Coomassie Blue and quantified by

using an Ortec 4310 densitometer. Background staining (EGTA control) was subtracted for each incubation mixture.

For trypsin treatment, the prewashed particulate fraction (8mg of protein) was preincubated with 100 μ l of buffer C (without phenylmethanesulphonyl fluoride) containing trypsin (5.36–2130 μ g/ml) and 1 mM-Ca²⁺. After incubation at 37°C for 15 min, a 2-fold molar excess of soya-bean trypsin inhibitor was added. Tubes were cooled to 4°C and centrifuged at 105000g for 30 min and washed with 8 ml of buffer C. The trypsin-treated particulate fraction was incubated as described above with 53.5 μ g of soya-bean trypsin inhibitor/ml, and Ca²⁺-dependent binding was measured.

Other procedures

The sedimentation coefficient was determined by the procedure of Martin & Ames (1961). Estimations of M_r and Stokes radius were obtained by chromatography on Sephacryl S-300 as described by Andrews (1964). The M_r was estimated from the Stokes radius and sedimentation coefficient by the procedure of Siegel & Monty (1966). Amino acid analysis was performed on 24 h hydrolysates treated with 6M-HCl at 110°C. Tryptophan was determined by the procedure of Bencze & Schmid (1957). Protein was measured by the procedure of Bradford (1976).

For immunological detection, proteins were transferred from SDS/9.5%-polyacrylamide gels to nitrocellulose paper at 12 V for 16 h by the procedure of Towbin *et al.* (1980). A 40 μ l sample ($A_{280} = 0.15$) of affinity-purified antibody was diluted in 50 ml of Dulbecco's phosphate-buffered saline (0.1 g of CaCl₂, 0.2 g of KCl, 0.2 g of KH₂PO₄, 0.1 g of MgCl₂·6H₂O, 8.0 g of NaCl and 2.16 g of Na₂HPO₄·7H₂O in 1 litre) containing 0.5% bovine serum albumin and 0.04% Nonidet P-40 and incubated for 1.5 h at 25°C with the paper. After treatment with peroxidase-coupled rabbit anti-sheep antibody in the above solution, colour was developed with *o*-dianisidine (25 μ g/ml) and H₂O₂ (0.01%).

Results

Comparison of control and EGTA extracts of the total particulate fraction from brain cortex by SDS/polyacrylamide-gel electrophoresis routinely identified four proteins which were unique to the EGTA extract, of M_r 68000, 34000, 32000 and 17000.

The four proteins were precipitated quantitatively by addition of (NH₄)₂SO₄ to 55% saturation and adjustment of the pH to 4.3. Two-dimensional gel electrophoresis of this fraction indicated that the unique bands represented predominantly

single proteins. The pI values of two proteins were estimated as 4.1 for the M_r -17000, 4.4–4.6 for the M_r -34000, 4.5–4.7 for the M_r -32000 and 4.9–5.1 for the M_r -68000. The M_r -17000-protein migrated as a doublet in the absence of added EGTA and was identified as calmodulin on the basis of its quantitative binding to fluphenazine-agarose and its ability to activate 3',5'-cyclic nucleotide phosphodiesterase of cardiac muscle.

The three proteins of M_r 68000, 34000 and 32000 were purified from bovine brain by salt fractionation at pH 4.3, DEAE-Sephacel and Affi-Gel Blue chromatography. Separation of the doublet of proteins of M_r 34000 and 32000 from that of M_r 68000 was accomplished by chromatography on Affi-Gel Blue-agarose (Fig. 1). The M_r -32000 and -34000 proteins were not retained by the column, and the M_r -68000 protein was eluted between 0.2 and 0.3 M-NaCl. At this stage, the M_r 68000-protein was over 98% pure and the doublet 70–85% pure by densitometric analysis. Since these proteins are enriched in the original extracts, purification was 15–20-fold over the EGTA extract. The scheme yielded 4–7 mg of M_r -68000 protein and a similar amount of the partially purified protein doublet per kg wet wt. of tissue.

Fig. 1. Affi-Gel chromatography of EGTA-extracted proteins

A dialysed protein sample (20.8 mg) of pooled fractions from DEAE-Sephacel chromatography was applied to an Affi-Gel Blue column (1.5 cm $\times$ 18 cm). The column was washed with 20 mM-Tris/HCl, pH 7.5, containing 2 mM-MgCl₂, 0.5 mM-dithiothreitol and 0.1 mM-EGTA (60 ml) and eluted with 300 ml of a 0–1.0 M gradient (---) of NaCl in the same buffer. The SDS/polyacrylamide-gel (6–15%) electrophoresis patterns, after staining with Coomassie Blue, of representative samples (60 μ l) from the elution are inserted directly above the fractions. Fractions (4 ml) from the column were collected and the A_{280} was monitored.

The concentration of protein doublet or M_r -68000 protein in brain cortex was estimated to be 20mg/kg.

One-dimensional peptide mapping of *S. aureus* protein digests of the three proteins are shown in Fig. 2. Bovine serum albumin was included for comparison. Peptide maps obtained by this technique did not demonstrate structural relatedness. In similar experiments, cleavage by CNBr or chymotrypsin failed to reveal common peptide fragments. The M_r -68000 protein was monomeric, on the basis of a M_r of 73800 calculated from the

Stokes radius and $s_{20,w}$ value (Table 1). The proteins of M_r 34000 and 32000 were also monomers, on the basis of gel-filtration experiments and $s_{20,w}$ values in the range 3.7–4.0S. The $A_{1\text{cm},280}^{1\%}$ for the M_r -68000 protein was 9.30. The amino acid composition of the M_r -68000 protein is given in Table 2.

Purified calmodulin, the M_r -68000 and the M_r -34000 and -32000 proteins were found to bind to the total particulate fraction of brain with half-maximal densitometric staining intensities at 3.5 μM , 8.3 μM and 150 μM - Ca^{2+} respectively (Fig. 3). Both the M_r -32000 and -34000 proteins followed the same binding curve. In similar

Fig. 2. One-dimensional peptide maps of bovine serum albumin and of M_r -68000, -34000 and -32000 proteins

A protein sample of 22 μg and bovine serum albumin (35 μg) were applied to a 6–15% gradient polyacrylamide slab gel. After brief staining with Coomassie Blue, the protein bands were cut out and placed on a second 15%-acrylamide gel with 0 (lanes a, d, g and j), 5 (lanes b, e, h and k) or 10 μg of *S. aureus* protease (lanes c, f, i and l)/ml. Samples included albumin (lanes a–c), M_r -68000 protein (lanes d–f), M_r -34000 protein (lanes g–i) and M_r -32000 protein (lanes j–l). The 15% polyacrylamide gel was stained for 18 h with Coomassie Blue.

Table 2. Amino acid composition of the M_r -68000 protein
Abbreviation: N.D., not determined.

Amino acid	Composition (mol%)
Leu	11.9
Val	5.0
Ala	8.7
Gly	6.8
Ile	6.4
Ser	6.3
Thr	5.8
Arg	6.2
Lys	8.0
His	1.8
Asx	10.6
Glx	12.9
Tyr	0.49
Phe	3.60
Trp	0.11
Met	2.5
Pro	2.9
Cys	N.D.

Table 1. Physical properties of bovine brain Ca^{2+} -dependent membrane-binding protein of M_r 68000
Numbers of determinations are shown in parentheses.

Property	Method	Value
M_r	SDS/polyacrylamide-gel electrophoresis	68000
M_r	Gel filtration	74000
Stokes radius (a)	Gel filtration	$3.54 \pm 0.14 \text{ nm}$ (5)
Sedimentation coefficient ($s_{20,w}$)	Sucrose density gradient	$5.06 \pm 0.26 \text{ S}$ (7)
M_r^*	Stokes radius and $s_{20,w}$	73800
Frictional ratio†		1.28

* Calculated by the equation:

$$M_r = 6\pi \cdot \eta_{20,w} \cdot s_{20,w} \cdot a \cdot N / (1 - \bar{v} \cdot \rho_{20,w})$$

where N = Avogadro's number, $\eta_{20,w}$ = viscosity of water at 20°C, $\rho_{20,w}$ = density of water at 20°C, and $\bar{v}$ = partial specific volume. A $\bar{v}$ of 0.725 ml/g was assumed (Siegel & Monty, 1966).

† Calculated by the equation (Siegel & Monty, 1966):

$$f/f_0 = a/(3\bar{v}M_r/4\pi N)^{\frac{1}{3}}$$

Fig. 3. Effect of Ca²⁺ concentration on binding of purified proteins to brain particulate fraction

The extracted and washed particulate fraction of brain (57 mg) was incubated with 19 μg of calmodulin (P17), 9.5 μg of the *M_r*-68000 protein (P68) and the purified fraction containing 19 μg of *M_r*-34000 and -32000 (P32) proteins. After incubation in buffer C containing 100 mM-NaCl and 200 μM-Ca²⁺-EGTA at different free Ca²⁺ concentrations for 20 min at 30°C, mixtures were diluted with incubation buffer and centrifuged at 105000g for 30 min. The pellets were extracted with 5 mM-EGTA and the supernatants subjected to SDS/polyacrylamide (6–15%) gel electrophoresis, followed by densitometric analysis against controls containing EGTA during incubation.

experiments, synergistic binding effects between calmodulin, *M_r*-68000 protein and the *M_r*-34000 and -32000 proteins were not observed. When the washed particulate fraction was pretreated with trypsin, Ca²⁺-dependent calmodulin binding was decreased, but binding of the *M_r*-68000 protein or the protein doublet remained within control values, with little decrease in binding owing to trypsin treatment (Fig. 4).

Co-electrophoresis and immunoblot analysis of the *M_r*-68000 protein of brain, calmodulin and gizzard *M_r*-67000 calcimedlin on SDS/9.5%-polyacrylamide gels is shown in Fig. 5. When compared in this system, the *M_r*-68000 protein of brain migrated slightly ahead of the *M_r*-67000 calcimedlin. Degradation products resulting from storage of the *M_r*-68000 brain protein were apparent (Fig. 5a, lane 4). The *M_r*-68000 protein of bovine brain did not cross-react with the anti-(*M_r*-67000 chicken calcimedlin) antibody (Fig. 5b), even though severalfold more *M_r*-68000 brain protein was applied and transferred compared with the *M_r*-67000 species of calcimedlins.

Fig. 4. Effect of trypsin treatment of brain particulate fraction on the binding of calmodulin and of *M_r*-68000 -34000 and -32000 proteins

The washed and extracted particulate fraction of brain (8.0 mg of protein) was treated with 5.36–2130 μg of trypsin/ml in buffer C (without phenylmethanesulphonyl fluoride) containing 1 mM-Ca²⁺ for 15 min at 37°C. A 2-fold molar excess of soya-bean trypsin inhibitor was added and the trypsin-treated particulate fraction isolated as described. This fraction was then incubated with 19 μg of calmodulin (P17; ●), 9.5 μg of the *M_r*-68000 protein (P68; ○) and the purified fraction containing 19 μg of *M_r*-34000 (P34; ■) and *M_r*-32000 (P32; □) proteins under conditions described in the legend of Fig. 3, with the addition of 53.5 μg of soya-bean trypsin inhibitor/ml and either 0.5 mM-Ca²⁺ or 150 μM-EGTA (control). Densitometric analysis of the supernatant of the EGTA-extracted pellets containing the bound proteins was then performed. The mean intensity (±s.d.) of four control incubations without trypsin treatment is also shown.

Discussion

The proteins isolated in this study occur in high concentration in brain cortex and exhibit specificity for Ca²⁺, since selective extraction with EGTA could be prevented by addition of excess Ca²⁺, but not Mg²⁺. Unlike calmodulin, the brain proteins were not found to possess high affinity for Ca²⁺ in aqueous solution, measured by equilibrium dialysis at 10 μM- and 50 μM-Ca²⁺. Ca²⁺-dependent

Fig. 5. SDS/polyacrylamide-gel electrophoresis and immunoblot detection of the M_r -68000 bovine brain protein and the M_r -67000 calcimedin

In (a) samples of standard rabbit longissimus dorsi myofibrils (22 μ g; lane 1), a mixture of gizzard calcimedins and calmodulin (19 μ g; lane 2), calmodulin alone (6 μ g; lane 3) and M_r -68000 brain protein (18 μ g; lane 4) were electrophoresed on a SDS/9.5%-acrylamide gel and stained with Coomassie Blue. The M_r values of protein standards are given in the left margin. In a similar experiment (b), immunoblots of proteins were performed by electrophoretic transfer to nitrocellulose paper and peroxidase-coupled staining by using the affinity-purified antibody to the M_r -67000 calcimedin. Samples were standard rabbit longissimus dorsi myofibrils (22 μ g; lane 1), gizzard calcimedins (16 μ g and 8 μ g; lanes 2 and 3) and bovine brain M_r -68000 protein (11 μ g; lane 4).

binding, however, may be analogous to extracellular proteins, which associate with membranes by interacting exclusively with the negatively charged polar head groups of phospholipids via Ca^{2+} (Pusey *et al.*, 1982).

Interestingly, the purified brain proteins do not interact significantly with calmodulin or fluphenazine affinity columns equilibrated with 0.5 mM- Ca^{2+} . Only a small proportion (less than 15%) of the M_r -68000 protein was bound by these affinity columns. This low affinity distinguishes these proteins from the calcimedins, which are routinely isolated by fluphenazine affinity chromatography (Moore & Dedman, 1982). The M_r -68000 protein does not cross-react with polyclonal antibody to bovine serum albumin, and appears distinct on the basis of amino acid analysis and physical properties. Further, the M_r -34000 and -32000 proteins

show no structural homology by one-dimensional peptide mapping (as described in Fig. 2) with the clathrin light chains of brain cortex.

Although a large group of proteins which bind to chromaffin-granule membranes of adrenal has been isolated by Ca^{2+} -dependent affinity chromatography (Creutz *et al.*, 1983), only three specific proteins have been routinely enriched and purified from brain by the scheme described the present paper. The same set of Ca^{2+} -regulated proteins appear to be consistently extracted from the particulate fraction of brain cortex, on the basis of one-dimensional peptide mapping of proteins isolated from different preparations.

The physical properties and amino acid analysis of the M_r -68000 protein are quite similar to values reported by Südhof *et al.* (1984). Insensitivity of binding to trypsin pretreatment of membranes and Ca^{2+} -dependent liposome binding are characteristic of the M_r -32000 protein of the electric organ of *Torpedo marmorata* designated as calelectrin (Walker, 1982). The three proteins of brain isolated in the present study appear identical with this family of ubiquitous mammalian proteins found in liver, brain and adrenal medulla (Südhof *et al.*, 1984). These proteins were shown to cross-react immunologically with each other and with the M_r -34000 protein of *Torpedo marmorata*. A cytoskeletal protein from bovine adrenal of M_r -36000 also shares antigenic relationships with the *Torpedo* calelectrin and with pp 36, a protein substrate of tyrosine kinases (Geisow *et al.*, 1984). During the present study, the purified M_r -68000 brain protein was found to degrade on storage at -20°C to give three distinct protein-staining bands of increasing staining intensity, corresponding to M_r 36000, 34000 and 32000. This finding, coupled with the immunological cross-reactivity found by Südhof *et al.* (1984), suggests that structural relationships between the brain proteins must be further investigated. Although Südhof *et al.* (1984) have suggested that calelectrins may be identical with calcimedins prepared from striated and smooth muscle (Moore & Dedman, 1982), results obtained with antibody to chicken gizzard M_r -67000 calcimedins do not support a common identity. The possibility that species difference could account for the lack of reactivity appears unlikely, in view of the reactivity of calcimedin antibody with proteins of bovine heart, rabbit longissimus dorsi muscle and cultured mammalian muscle cells (Moore *et al.*, 1982, 1984).

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