Identification of residues essential for catalysis and binding of calmodulin in rat brain inositol 1,4,5-trisphosphate 3-kinase

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In order to identify the amino acid residues involved in calmodulin (CaM) binding and catalytic activity, rat brain inositol 1,4,5-trisphosphate (InsP₃) 3-kinase was expressed in *Escherichia coli* as a β -galactosidase fusion protein [clone C5; Takazawa, Vandekerckhove, Dumont & Erneux (1990) *Biochem. J.* 272, 107–112]. Three deletion mutants in the plasmid of clone C5 were generated using convenient restriction enzymes. The results show that the removal of 34 amino acids from the C-terminal end of InsP₃ 3-kinase resulted in an inactive protein which still interacted with CaM–Sepharose in a Ca²⁺-dependent way. The catalytic domain is thus located at the C-terminal end of the protein. A series of 5' deletion mutants was prepared and used to produce proteins with the same C-terminal end but shortened N-termini, varying in length by over 80 amino acids. Assay of InsP₃ 3-kinase activity in bacterial extracts indicated that a maximum of 275 amino acids in the C-terminal region may be sufficient for the construction of a catalytically active domain. Affinity chromatography on CaM–Sepharose of 5' and 3' deletion mutants revealed that the sequence stretching from Ser-156 to Leu-189 is involved in CaM binding and enzyme stimulation.

INTRODUCTION

An increase in cellular inositol 1,4,5-trisphosphate (InsP₃) concentration has been shown to mobilize intracellular Ca²⁺ (Berridge & Irvine, 1989). InsP₃ 3-kinase catalyses the phosphorylation of InsP₃ to inositol 1,3,4,5-tetrakisphosphate (InsP₄; Irvine *et al.*, 1986), which interacts with putative receptors (Bradford & Irvine, 1987; Donie & Reiser, 1989) to release intracellular Ca²⁺. InsP₄ appears to synergize with InsP₃, as initially proposed by Irvine and colleagues (Irvine *et al.*, 1988). Studies using mouse lacrimal cells (Morris *et al.*, 1987a; Changya *et al.*, 1989), hepatocytes (Joseph *et al.*, 1987) and sea-urchin eggs (Irvine & Moor, 1986) have indicated that InsP₄, together with a Ca²⁺-mobilizing inositol trisphosphate, can stimulate extracellular Ca²⁺ entry or Ca²⁺ mobilization from intracellular pools which are not accessible to InsP₃ alone (Cullen *et al.*, 1990). Other studies in rat cerebellum (Joseph *et al.*, 1989) and adrenal cortical microsomes (Ely *et al.*, 1990) and also in neuroblastoma cells (Gawler *et al.*, 1990) have provided evidence for a direct second messenger function of InsP₄ in the release of Ca²⁺.

Ca²⁺ is a potent stimulator of InsP₃ 3-kinase in lysed cell preparations, e.g. RINm5F cells (Biden & Wollheim, 1986), mouse thymocytes (Zilberman *et al.*, 1987) and rat hepatocytes (Biden *et al.*, 1988), within the physiological range of free Ca²⁺ concentrations (0.1–1 μ M). Ca²⁺ also positively regulates InsP₄ production: an increase in cytosolic free Ca²⁺ induces high levels of InsP₄ in many cell types (e.g. Zilberman *et al.*, 1987).

The complex formed by Ca²⁺ and calmodulin (CaM) appears to be an activator of InsP₃ 3-kinase (Biden *et al.*, 1987; Morris *et al.*, 1987b; Ryu *et al.*, 1987; Yamaguchi *et al.*, 1988; Takazawa *et al.*, 1988), and this interaction has been used to purify the enzyme by affinity chromatography on CaM–Sepharose (Takazawa *et al.*, 1989; Lee *et al.*, 1990). The purified enzyme from rat brain was associated with a 50 kDa protein doublet on SDS/PAGE (Takazawa *et al.*, 1990a). cDNAs encoding a 50 kDa InsP₃ 3-kinase have been isolated and sequenced (Takazawa *et al.*, 1990b; Choi *et al.*, 1990) and a cDNA clone was expressed

in *Escherichia coli* as a β -galactosidase fusion protein containing 430 amino acids. In the present study, a series of deletion mutants was prepared in order to identify the amino acids involved in CaM binding to and catalytic activity of InsP₃ kinase. A model of the structural organization of the catalytic and CaM-binding domains of the protein is proposed.

MATERIALS AND METHODS

Endonuclease deletion mutants

Bluescript plasmids harbouring cDNA inserts encoding rat brain InsP₃ 3-kinase (C5 and CP16; Takazawa *et al.*, 1990b) were isolated from a cDNA library. DNA prepared from C5 was digested with *Sph*I and *Bal*I to delete 544 and 89 bp respectively to the 3' side of the insert, giving C5-S and C5-B respectively. Another construct was made with *Hinc*II, which cuts restriction sites both at position 318 of the insert and 3' in the polylinker, to delete 1299 bp (giving C5-H). After ligation, DNA was used to transform XL1-Blue competent cells. This step created artificial translation stop codons following the point of deletion after 66, 6 and 36 bp for *Sph*I, *Bal*I and *Hinc*II respectively. The newly constructed plasmids were expressed as β -galactosidase fusion proteins and identified on blots by immunodetection (Takazawa *et al.*, 1990a) using anti-(rat brain 50 kDa protein) serum. In some experiments antibodies were affinity-purified using recombinant InsP₃ 3-kinase adsorbed to a nitrocellulose filter (Sambrook *et al.*, 1989).

Exonuclease III/mung bean nuclease deletions

Exonuclease III/mung bean nuclease deletion mutants were prepared using the Stratagene Exonuclease III/Mung Bean Nuclease Deletion kit by following the supplier's instructions. For unidirectional deletion starting from the 3' end of the insert, 5 μ g of C5 plasmid DNA was digested with *Bgl*II and *Apa*I restriction enzymes. Exonuclease III digested 5' single-strand overhangs created by *Bgl*II at position 1184 of the insert. Incubation with exonuclease was at 30 °C from 3 to 7 min;

Abbreviations used: CaM, calmodulin; PMSF, phenylmethanesulphonyl fluoride.

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MTLPGHPTGMARPRGAGPCSPGLERAPRRSVGE 33
LRLLEFARCAAVAAAAAAGEPRARGAKRRGGQVNG 69
LPRAAPAPVIPQLTVTSEEDVAPASPGPPDREGNWL 105
PAAGSHLQQPRLSTSSLSSTGSSSLEDSEDDLLS 141
DSESRSRGNVQLETSEDVGGKS HWOKIRTMVNL PVM 177
SPFKKRYSWVQLAGHTGSFKAAGTSGILIKRSSEPE 213
HYCLVRLMADVLRGCV PAFHGVVERDGE SYLQLQDL 249
LDGFDGPGCVLDCKMGVVRTYLEEELTK ARERPKLRKD 285
MYKKMLAV DPEAPTEEEHAQRAVTKPRYMQWREGIS 321
SSTTLGFRIEGIKKADGSCSTDFKTTRSREQVTRVF 357
EEFMQGD AEVLKRYLNRLQOIRDTLE ISDFFRHEV 393
IGSSLLFV HDHCHRRAGVNLIDFG KTTPLPDGQILDH 429
RRPWEEGNREDGYLLGLDNLIGILANLAER 459

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Fig. 1. Amino acid sequence of recombinant *InsP₃* 3-kinase

Putative CaM-binding sites based on secondary structure analysis are underlined. The probable CaM-binding domain based on deletion mutant analysis is doubly underlined.

aliquots were taken from the incubation mixture at intervals of 1 min. Each sample was treated with mung bean nuclease to create blunt ends, ligated to recircularize the plasmid and used to transform XL1-Blue competent cells. DNA was isolated from individual colonies and digested with *EcoRI* and *KpnI* to map the extent of the deletions. The two newly prepared constructs were sequenced (Sanger *et al.*, 1977) to determine the extent and the 3' end of each deletion. Both 4M1 and 5M1 had in-frame stop codons 18 bp downstream from Gly-240 and Leu-189 respectively (Fig. 1). For unidirectional deletion starting from the 5' end of the insert, 5 µg of plasmid DNA from CP16 was digested with *XbaI*, which cleaved at a unique restriction site which is only present in the Bluescript polylinker. The 5' restriction overhang was filled in with deoxythio-derivatives, and digestion was carried out with *SmaI* at position 319 of the insert followed by exonuclease III from 1.5 to 5.5 min of incubation. Samples were then treated as above. Plasmid DNA of individual colonies was digested with *BglII* and *SstI* to map the extent of the deletions. The colonies obtained by exonuclease deletion starting from the 5' end were tested for expression of β -galactosidase-*InsP₃* 3-kinase fusion protein, as verified by *InsP₃* 3-kinase assay, immunodetection and DNA sequencing. The deleted cDNAs which were in-frame with the β -galactosidase part of Bluescript were kept for further analysis. The molecular masses of immunoreactive products are consistent with the predicted sizes of the proteins calculated from their sequences.

Expression of *InsP₃* 3-kinase and related mutants

To express proteins from deleted constructs, LB medium (5 ml) containing 50 µg of ampicillin/ml was inoculated for an overnight incubation with a single colony containing the Bluescript plasmid at 37 °C. After the addition of isopropyl β -thiogalactoside (1 mM final concentration) for 4 h, the bacteria were harvested by centrifugation and resuspended in 0.5 ml of cold lysis buffer [50 mM-Tris/HCl (pH 8), 1 mM-EDTA, 0.2 mM-phenylmethanesulphonyl fluoride (PMSF), 2.5 µM-leupeptin, 10 µg of trypsin inhibitor/ml, 10% sucrose, 12 mM-2-mercaptoethanol, and 1% Triton X-100]. After 5 min at 0 °C followed by centrifugation (15000 g, 5 min) the pellet was resuspended in 0.5 ml of the same buffer, except that Triton was replaced by 0.5% SDS, and the 2-mercaptoethanol concentration was 5%. After 5 min at 0 °C and centrifugation, the supernatant was used either to measure *InsP₃* 3-kinase activity or for SDS/PAGE on 10–13% polyacrylamide gels and immunodetection. Bacterial lysis and expression of recombinant kinase in 0.5% SDS gave greater expression compared with the procedure

of Takazawa *et al.* (1990b), as verified by assay of *InsP₃* 3-kinase activity and/or immunodetection.

CaM-Sepharose affinity chromatography

Bacterial lysate (40 µl) was diluted in 2 ml of 20 mM-Tris/HCl (pH 7.5)/0.2 mM- CaCl_2 /0.1 M-NaCl/1% Triton X-100/12 mM-2-mercaptoethanol/0.4 mM-PMSF/5 µM-leupeptin (protease inhibitors) and immediately applied to a 0.4 ml CaM-Sepharose column. The column was washed with 5 ml of 20 mM-Tris/HCl (pH 7.5)/0.2 mM- CaCl_2 /0.4 M-NaCl/0.5% Triton X-100/12 mM-2-mercaptoethanol/protease inhibitors, and CaM-binding proteins were eluted in 1.5 ml of 20 mM-Tris/HCl (pH 7.5)/2 mM-EGTA/0.4 M-NaCl/0.1% Triton X-100/12 mM-2-mercaptoethanol/protease inhibitors. In general, 50 µl of the EGTA eluate was used for SDS/PAGE and immunodetection.

Analytical procedures

InsP₃ 3-kinase activity was measured as previously described (Takazawa *et al.*, 1988) in 100 µl of medium containing 10 µM free Ca^{2+} or 1 mM-EGTA, 84 mM-Hepes/NaOH (pH 7.5), 1 mg of BSA/ml, 1 mM-ATP, 20 mM- MgCl_2 , 12 mM-2-mercaptoethanol, 0.1 µM-CaM, diluted enzyme solution, [^{32}P]*InsP₃* (200–250 Ci/mmol; New England Nuclear) and unlabelled *InsP₃* to a final concentration of 10 µM. Protein concentration was measured according to Bradford (1976).

RESULTS

Construction of deleted cDNAs encoding *InsP₃* 3-kinase

A cDNA clone encoding the C-terminal part of the *InsP₃* 3-kinase was initially isolated by immunoscreening of a rat brain cDNA library (clone C5 in Takazawa *et al.*, 1990b). When expressed in *E. coli* as a β -galactosidase fusion protein, the clone showed Ca^{2+} /CaM-sensitive *InsP₃* 3-kinase activity. To localize the catalytic and CaM-binding domains, further cDNAs encoding truncated *InsP₃* 3-kinase were constructed. For example, C-terminal segments of the coding sequence were deleted using two restriction enzymes cutting only in the insert (*BalI* and *SphI*). *HincII* restriction sites were present at position 318 in the insert and also at a unique 3' position in the polylinker. Deletion mutants (3') were generated which truncated the recombinant kinase from the C-terminal end; 5' deletion mutants were obtained using a full-length *InsP₃* 3-kinase clone (referred to as CP16 in Takazawa *et al.*, 1990b). Fig. 2 summarizes the various constructs that were obtained.

Characterization of 3' deletion mutants

In contrast to C5, the three clones with deletions (C5-B, C5-S and C5-H), and a lysate derived from a non-recombinant clone, did not have *InsP₃* 3-kinase activity after expression (Table 1). The proteins in bacterial lysates were analysed on immunoblots using affinity-purified antibodies against *InsP₃* 3-kinase (Fig. 3). The signals were compared before and after elution from CaM-Sepharose columns to establish whether or not the expressed 3-kinases contained a CaM-binding domain. The antibodies reacted with 46 kDa and 36 kDa bands in lysates derived from C5-B and C5-S respectively, both before and after CaM-Sepharose. In contrast, the antibodies recognized a 22 kDa band in a C5-H lysate, but the signal was not seen after elution from the affinity column. Thus the deletion of 34 amino acids from the C-terminal end of the *InsP₃* 3-kinase is sufficient to abolish *InsP₃* 3-kinase activity. In addition, the protein sequence stretching from amino acid 172–304 may include a CaM-binding domain required for stimulation by Ca^{2+} /CaM.

To confirm the data obtained previously, two 3' deletion mutants were obtained by digestion of C5-prepared DNA with

Fig. 2. InsP_3 3-kinase cDNA constructs used in this study

The InsP_3 3-kinase portion of the expressed protein from C5 had 394 residues and started at Val-66 of the entire protein (see Fig. 1). Plasmid designations are shown on the left. Enzyme activity (if present) and CaM sensitivity are taken from Table 1. CaM binding is assessed by CaM-Sepharose affinity chromatography and immunoblotting (Figs. 3-5). A proposed model of the structural organization of catalytic and CaM-binding domains of the protein is shown.

Fig. 3. Western blot of deletion mutants C5-B, C5-S and C5-H

Crude bacterial lysate (1 μl ; lanes a) or 50 μl of the EGTA eluate from a CaM-Sepharose affinity column (lanes b) was applied to SDS/12% PAGE. Immunoblot analysis was performed using affinity-purified antibody to C5. NR, bacterial lysate from a non-recombinant clone. The arrows show the immunoreactive bands.

exonuclease III. As expected, the truncated expressed proteins 4M1 and 5M1 did not have InsP_3 3-kinase activity (Table 1). They did, however, bind to CaM-Sepharose, as shown by immunoblot analysis (Fig. 4), indicating that residues 172-189 are important for CaM binding.

Table 1. Enzymic activity of deletion mutants of InsP_3 3-kinase

InsP_3 3-kinase activity was determined at 10 μM - InsP_3 in the presence of 0.1 μM -CaM and 10 μM free Ca^{2+} or 1 mM-EGTA for basal activity using crude bacterial extracts from individual clones. n.d., activity was undetectable. Results are means $\pm$ S.D. of triplicate determinations.

Clone	InsP_3 3-kinase activity (nmol/min per mg of protein)		Stimulation by CaM (fold)
	+ EGTA	+ Ca^{2+} /CaM	
Non-recombinant	n.d.	n.d.	-
C5	47 $\pm$ 11	142 $\pm$ 13	3.0
C5-B	n.d.	n.d.	-
C5-S	n.d.	n.d.	-
C5-H	n.d.	n.d.	-
4M1	n.d.	n.d.	-
5M1	n.d.	n.d.	-
1.5M1	348 $\pm$ 13	979 $\pm$ 73	2.8
3.5M4	427 $\pm$ 36	959 $\pm$ 41	2.2
3.5M7	124 $\pm$ 6	969 $\pm$ 24	7.8
3.5M3	284 $\pm$ 15	276 $\pm$ 19	1.0
3.5M6	207 $\pm$ 55	196 $\pm$ 31	0.9
4.5M4	790 $\pm$ 38	829 $\pm$ 6	1.0
4.5M1	n.d.	n.d.	-
5.5M3	n.d.	n.d.	-

Fig. 4. Western blot of 3' deletion mutants

Immunodetection was performed with non-purified antiserum. The two 3' deletion mutants were compared with C5-expressed InsP_3 3-kinase (indicated by arrows). Lanes a, crude bacterial lysate; lanes b, EGTA eluate from CaM-Sepharose column.

Characterization of 5' deletion mutants

As C5-B, which harbours a deletion of 34 amino acids at the C-terminal end of InsP_3 3-kinase, had no enzymic activity, other 5' deletion mutants were prepared to determine the position of the catalytic domain. Fig. 2 shows the DNAs which were deleted to various extents to obtain mutants within the putative CaM-binding domain. Proteins expressed using 1.5M1, 3.5M4 and 3.5M7 had the same properties as C5, i.e. they were enzymically active and CaM-sensitive. The activities of the mutants and of C5 were greatly different (Table 1). We do not yet know why each mutant is expressed at different levels; proteolysis, culture conditions or the loss of a regulatory domain may be possible causes of the difference. Further deletions resulted in 3.5M3, 3.5M6 and 4.5M4; the expressed proteins were active but CaM-insensitive, suggesting that kinase activity and interaction with

Fig. 5. Western blot of 5' deletion mutants

Experimental conditions are the same as in Fig. 3. Plasmid designations are defined in Fig. 2.

CaM are associated with different domains of the protein. Finally, deletions 4.5M1 and 5.5M3 were both enzymically inactive (Fig. 2 and Table 1). Therefore a maximum of 275 amino acids in the C-terminal region may be sufficient for the construction of a catalytically active domain. A protein sequence stretching from Ser-156 to Val-172 was shown to be important in CaM binding. This conclusion is supported by immunoblot analysis (Fig. 5): purified antibodies reacted with a 34 kDa band from expressed 3.5M7 both before and after chromatography on CaM-Sepharose. In contrast, antibodies only recognized a 33 kDa band before chromatography on CaM-Sepharose, and no bands in an EGTA eluate in lysates derived from 3.5M3.

DISCUSSION

Little is known about the structural organization of InsP_3 3-kinase. Studies with bovine brain InsP_3 3-kinase have suggested that CaM-Ca^{2+}_3 and CaM-Ca^{2+}_4 are the biologically active species involved in the ternary complex of the stimulated enzyme (Li *et al.*, 1989). Johanson *et al.* (1988) have provided evidence that the purified enzyme behaved as a monomer, and eventually as a dimer when mixed with Ca^{2+} /CaM and chromatographed on a Superose 12 column. Our data do not exclude this hypothesis, although it is possible that in the presence of Ca^{2+} /CaM the enzyme may interact with other proteins due to hydrophobic interactions. In other studies, limited proteolysis of the native rat brain enzyme by trypsin has indicated that the limit size of the enzyme that would contain both CaM-binding and catalytic domains is about 36 kDa (Takazawa *et al.*, 1990b).

Recent molecular cloning and expression of InsP_3 3-kinase has provided the means for identifying residues that are essential for catalysis and binding of CaM, as well as allowing structural comparison with other CaM-regulated enzymes. CaM-binding sites are not sequence-specific; CaM recognizes basic amphiphilic α -helices with clusters of positively charged and hydrophobic amino acids (O'Neil & DeGrado, 1990). This may provide a rather simple and versatile structural motif that permits many interactions with a wide variety of proteins. In InsP_3 3-kinase, five segments of amino acids were tentatively identified as CaM-interacting sites (Fig. 1). Depending on the protein, the CaM-binding sequences are positioned in different parts of the sequence: (1) close to the C-terminal end (e.g. in myosin light chain kinase; Bagchi *et al.*, 1989), (2) very close to the N-terminal end (e.g. in 61 or 59 kDa isoenzymes of CaM-sensitive cyclic

nucleotide phosphodiesterase; Beavo & Reifsnnyder, 1990), or (3) in the middle of the protein (e.g. the α -subunit of CaM-kinase II; Lin *et al.*, 1987).

Results obtained with C-terminal mutants of InsP_3 3-kinase reveal that the removal of only 34 amino acids from the C-terminal end resulted in an inactive protein which still interacted with CaM-Sepharose in a Ca^{2+} -dependent way. Thus the C-terminal region is essential for catalytic function but not for CaM binding. By comparing the results of 3' and 5' deletion mutants, 275 amino acids in the C-terminal region are shown to be required for catalytic activity. The InsP_3 - and ATP-binding sites are thus located at the C-terminal end, in contrast to the InsP_3 receptor, in which the N-terminus contains the ligand-binding domain (Mignery & Sudhof, 1990). The domain organization of InsP_3 3-kinase is also different from the α -subunit of CaM kinase II, in which the protein kinase domain is found in the N-terminal end (amino acid residues 1 to 220–250 for a 50 kDa protein; Lin *et al.*, 1987). Since CaM kinase is a holoenzyme (300–700 kDa) composed of subunits of 49–62 kDa, and InsP_3 3-kinase is apparently active as a monomer, the two protein structures are thus very different.

Fig. 6. Proposed secondary structure of InsP_3 kinase

(a) Helix wheel representation of the putative CaM-binding sequence H(164)WQKIRTMVNL(174). The sequence is drawn as proposed by O'Neil & DeGrado (1990). Hydrophobic residues are circled and positively charged residues are denoted by a +. (b) The helix is shown in two dimensions. The repeat unit extends for 0.54 nm, with 3.6 amino acid residues per turn. Both hydrophobic and positively charged regions are indicated.

The localization of the CaM-binding domain resulted from the comparison of both 3' and 5' deletion mutants (Fig. 2). Mutants produced by 3' deletion and C5-H mutant led us to conclude that amino acids Val-172–Leu-189 are important for CaM binding. Comparison of expressed 3.5M7 and 3.5M3 proteins shows the importance of residues Ser-156–Val-172 for CaM binding. Taken together, these results indicate that the sequence between Ser-156 and Leu-189 may contain the CaM-binding domain. Secondary structure predictions are consistent with this result, as CaM recognizes positively charged amphiphilic α -helices, often containing a tryptophan residue (Glaser *et al.*, 1989). Sequence analysis showed that amino acids His-164–Leu-174 would meet these criteria: there is a high probability of helix formation, using the method of Chou & Fasman (1978) for secondary structure prediction, and clusters of hydrophobic residues are present adjacent to the basic His and Lys residues (Fig. 6). Interestingly, a proline residue at 175 (i.e. at the C-terminal end of the proposed sequence) will break the α -helix. Altogether, our data and sequence analysis suggest that amino acid residues His-164–Leu-174 constitute the main CaM-binding domain of the protein.

The identification of a CaM-binding domain raises the question of whether the InsP_3 3-kinase contains an autoinhibitory domain close to the CaM-binding site, perhaps at the N-terminal end. Regulation of many protein kinases by autoinhibitory domains has been reported, e.g. for calcineurin or cyclic nucleotide protein kinase (see Soderling, 1990, for review). According to this model, the binding of the allosteric activator disrupts the interaction between autoinhibitory and catalytic domains, and the possibility of such a situation with InsP_3 3-kinase merits further study.

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REFERENCES

- Bagchi, I. C., Kemp, B. C. & Means, A. R. (1989) *J. Biol. Chem.* **264**, 15843–15849
- Beavo, J. A. & Reifsnnyder, D. H. (1990) *Trends Pharmacol. Sci.* **11**, 150–155
- Biden, T. J. & Wollheim, C. B. (1986) *J. Biol. Chem.* **261**, 11931–11934
- Biden, T. J., Comte, M., Cox, J. A. & Wollheim, C. B. (1987) *J. Biol. Chem.* **262**, 9437–9440
- Biden, T. J., Altin, J. G., Karjalainen, A. & Bygrave, F. L. (1988) *Biochem. J.* **256**, 697–701
- Berridge, M. J. & Irvine, R. F. (1989) *Nature (London)* **341**, 197–205
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Bradford, P. G. & Irvine, R. F. (1987) *Biochem. Biophys. Res. Commun.* **149**, 680–685
- Changya, L., Gallacher, D., Irvine, R. F., Potter, B. V. L. & Petersen, O. H. (1989) *J. Membr. Biol.* **109**, 85–93
- Choi, K. Y., Kim, H. K., Lee, S. Y., Moon, K. H., Sim, S. S., Kim, J. W., Chung, H. K. & Rhee, S. G. (1990) *Science* **248**, 64–66
- Chou, P. Y. & Fasman, G. J. (1978) *Adv. Enzymol.* **47**, 45–148
- Cullen, P. J., Irvine, R. F. & Dawson, A. P. (1990) *Biochem. J.* **271**, 549–553
- Donie, F. & Reiser, G. (1989) *FEBS Lett.* **254**, 155–158
- Ely, J. A., Hunyady, L., Baukal, A. J. & Catt, K. (1990) *Biochem. J.* **268**, 333–338
- Gawler, D. J., Potter, B. V. & Nahorski, S. R. (1990) *Biochem. J.* **272**, 519–524
- Glaser, P., Elmoaglou-Larazidou, A., Krin, E., Ladant, D., Barzu, O. & Danchin, A. (1989) *EMBO J.* **8**, 967–972
- Irvine, R. F. & Moor, R. M. (1986) *Biochem. J.* **240**, 917–920
- Irvine, R. F., Letcher, A. J., Lander, D. J. & Berridge, M. J. (1986) *Nature (London)* **320**, 631–634
- Irvine, R. F., Moor, R. M., Pollock, W. K., Smith, P. M. & Wreggett, K. A. (1988) *Philos. Trans. R. Soc. London Ser. B* **320**, 281–298
- Johanson, R. A., Hansen, C. A. & Williamson, J. R. (1988) *J. Biol. Chem.* **263**, 7465–7471
- Joseph, S. K., Hansen, C. A. & Williamson, J. R. (1987) *FEBS Lett.* **219**, 125–129
- Joseph, S. K., Hansen, C. A. & Williamson, J. R. (1989) *Mol. Pharmacol.* **36**, 391–397
- Lee, S. Y., Sim, S. S., Kim, J. W., Moon, K. H., Kim, J. H. & Rhee, S. G. (1990) *J. Biol. Chem.* **265**, 9434–9440
- Li, G., Comte, M., Wollheim, C. & Cox, J. A. (1989) *Biochem. J.* **260**, 771–775
- Lin, C. R., Kapiloff, M. S., Durgerian, S., Tanemoto, K., Russo, A. F., Hanson, P., Schulman, H. & Rosenfeld, M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5962–5966
- Mignery, G. A. & Sudhof, T. C. (1990) *EMBO J.* **9**, 3893–3898
- Morris, A. P., Gallacher, D. V., Irvine, R. F. & Petersen, G. H. (1987a) *Nature (London)* **330**, 653–655
- Morris, A. J., Downes, C. P., Harden, T. K. & Michell, R. H. (1987b) *Biochem. J.* **248**, 489–493
- O'Neil, K. T. & DeGrado, W. F. (1990) *Trends Biochem. Sci.* **15**, 59–64
- Ryu, S. H., Lee, S. Y., Lee, K. Y. & Rhee, S. G. (1987) *FASEB J.* **1**, 388–393
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- Soderling, T. R. (1990) *J. Biol. Chem.* **265**, 1823–1826
- Takazawa, K., Passareiro, H., Dumont, J. E. & Erneux, C. (1988) *Biochem. Biophys. Res. Commun.* **153**, 632–641
- Takazawa, K., Passareiro, H., Dumont, J. E. & Erneux, C. (1989) *Biochem. J.* **261**, 483–488
- Takazawa, K., Lemos, M., Delvaux, A., Lejeune, C., Dumont, J. E. & Erneux, C. (1990a) *Biochem. J.* **268**, 213–217
- Takazawa, K., Vandekerckhove, J., Dumont, J. E. & Erneux, C. (1990b) *Biochem. J.* **272**, 107–112
- Yamaguchi, K., Hirata, M. & Kuriyama, H. (1988) *Biochem. J.* **251**, 129–134
- Zilberman, Y., Howe, L. R., Moore, J. P., Hesketh, T. R. & Metcalfe, J. C. (1987) *EMBO J.* **6**, 957–962

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