MOLECULAR CLONING OF
THE cDNA ENCODING β-CELL
CALCIUM/CALMODULIN-DEPENDENT
PROTEIN KINASE II

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INTRODUCTION

Current hypotheses postulate that the metabolism of glucose within islets results in a transient increase in the ATP:ADP ratio, leading to depolarization and opening of voltage-dependent Ca²⁺ channels, promoting influx of Ca²⁺ into the β-cell. The increase in intracellular calcium may activate Ca²⁺-dependent protein kinases, which in turn phosphorylate key components in the secretory machinery. The nature and identity of these components are at present unclear. In addition, the β-cell contains a high (15–50 µM) concentration of calmodulin (CaM) and it has been shown that inhibitors of CaM block insulin secretion. Possible candidates for Ca²⁺/calmodulin-dependent protein kinases acting as regulatory enzymes in the exocytotic release process are phosphorylase kinase, myosin light chain kinase and CaM kinases.

Ca²⁺/calmodulin-dependent protein kinase II (CaMkII) is a multifunctional kinase that has been implicated in the regulation of a variety of cellular processes, including contractility, gene expression and neurotransmitter release and synthesis. It has been purified and characterized from mammalian brain and several nonneuronal tissues and from Drosophila. Molecular cloning indicates that at least four distinct genes encode CaMkII isoforms: α, β, γ and δ subunits and their variants originated by alternative splicing of the gene transcripts. The α and β subunits are expressed primarily in brain and the γ and δ are the predominant forms in peripheral tissues. The kinase is a multimer of 4–12 subunits, with subunit size ranging from 51–60 kDa on SDS-PAGE, depending on the isoform and tissue. CaMkII autophosphorylates in its autoinhibitory domain in the presence of Ca²⁺, calmodulin and MgATP. Autophosphorylation at this site converts the kinase to a Ca²⁺-independent or autonomous enzyme.
Support for a role of a β-cell CaM kinase (CaMk) in the secretory process is provided by evidence that alloxan, a diabetogenic agent, and KN-62, a specific inhibitor of CaMkII inhibit both glucose-induced insulin secretion and CaMkII activity

In order to identify the β-cell CaMk and its relationships to CaMkII, we have investigated: a) the expression of mRNA for CaMkII in the rat β-cell line RINm5F by Northern analysis; b) the aminoacid homology of the enzyme expressed in rat islets with brain CaMkII by immunoreactivity; c) the Ca\(^{2+}\)/calmodulin-dependent phosphorylation activity of islet extracts; d) the inhibition of β-cell CaMk by KN-62 and e) the expression of CaMkII in rat pancreatic islets by screening a cDNA library.

Since the characterization of β-cell CaMk will ultimately involve purification to homogeneity and comparison of kinetic and structural properties of this enzyme with those of CaMkII, we have used the baculovirus expression system to purify mg amounts of a subunit of brain CaMkII and have isolated a clone from a rat islet cDNA library.

**MATERIALS AND METHODS**

**Autophosphorylation Assay**

Isolated rat islets were lysed by sonication in 50 mM HEPES pH 7.5–1% NP-40 and incubated for 3 min at room temperature with 0.5 pmol of \([\gamma-^{32}P]-ATP\) (5000 Ci/mmol) in final concentrations of 10 mM MgCl\(_2\); 25 mM HEPES pH 7.5; 10 mM CaCl\(_2\) and 20 μM calmodulin (CaM), in a reaction volume of 100 μl. Parallel assays were conducted in the absence of Ca\(^{2+}\) and CaM, and with the addition of 1 mM EGTA; 1 mM trifluoperazine (TFP), and 30 μM KN62, individually or in combination. The reaction was terminated by the addition of 0.25 vol. of 5X dissociation buffer (250 mM Tris-Cl pH 6.8; 10% SDS; 5% β-mercaptoethanol; 50% glycerol and 0.5% bromophenol blue) and heating at 100°C for 3 min. The reaction products were resolved by SDS-PAGE, transferred to PVDF membranes for immunoanalysis and subsequently subjected to autoradiography.

**Immunoblot Analysis**

A synthetic peptide representing residues 281–309 of the α-subunit of brain CaMkII (which is identical in the α, β, γ and δ isozymes with the exception of 3 residues) was used to raise rabbit polyclonal antibodies. The specificity of the immune serum was tested by reaction with affinity purified mouse brain CaMkII α-subunit, expressed in a baculovirus system.

The products of the phosphorylation assay of rat islet proteins were resolved by SDS-PAGE and the proteins electroblotted onto PVDF membranes (Immobilon, Millipore). The membranes were soaked in blocking solution (5% nonfat dry milk; 0.02% Tween-20) for 1 hour and subsequently incubated for 12 hours at 4°C with the anti-CaMkII antibody in blocking solution. After washing steps in PBS-0.02% Tween-20 the membranes were incubated with alkaline phosphatase conjugated anti-rabbit IgG and immune complexes were detected by the addition of the chromogenic substrate NBT/BCIP.

**Northern Hybridization Analysis**

Total RNA extracted from RINm5F β-cells was resolved in a 1% methylmercury agarose gel, transferred and fixed to a nylon membrane (Hybond N, Amersham). A cDNA