氏　　名	畠山　裕康
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論文審査委員	主　査　教授　箕越　靖彦 教授　岡田　泰伸 教授　富永　真琴 教授　寺川　進（浜松医科大学） 教授　河西　春郎（東京大学）

論文内容の要旨

Rapid glucose sensing by protein kinase A for insulin exocytosisin mouse pancreatic islets

Glucose is the most important physiological regulator of insulin secretion from β cells of islets of Langerhans. Islet β cells rapidly take up and metabolize glucose, resulting in an increase in the cytosolic concentration of ATP within 1 min. Such increases in ATP concentration induce the closure of ATP-sensitive K^+ (K_{ATP}) channels and consequent depolarization of the cell membrane, again within a few minutes of glucose application. Depolarization of the cell membrane to a voltage of > −50 mV results in activation of voltage-dependent Ca^{2+} channels and an increase in $[Ca^{2+}]_i$ that triggers insulin exocytosis. The K_{ATP} channels and Ca^{2+}-dependent mechanism are thought to play a central role in glucose sensing for insulin exocytosis. Although additional mechanisms of glucose sensing have been proposed to coexist, their relative importance has remained unclear.

Exocytosis in many secretory cell types and neurons is regulated by both Ca^{2+} and cAMP. Their research group has previously shown that cytosolic cAMP potentiates Ca^{2+}-dependent insulin exocytosis (CIE) in β cells (Takahashi N. *et al. Proc. Natl. Acad. Sci. U.S.A.* **96**, 960, 1999). In these studies, individual β cells were subjected to whole-cell patch clamping and stimulated with large increases in $[Ca^{2+}]_i$ induced by photolysis of a caged-Ca^{2+} compound, thereby bypassing the K_{ATP} channel-dependent mechanism. They found that CIE was augmented by cAMP in a manner dependent on protein kinase A (PKA) and cytosolic ATP. It was not possible to study the action of extracellular glucose under the whole-cell clamp conditions, however, and it has remained unknown whether PKA contributes to glucose-induced insulin exocytosis (GIE). Inhibitors of PKA have been shown to have relatively small inhibitory effects on GIE in previous studies, in which exocytosis was measured over a long period without separation into the first and second phases.

They therefore subsequently developed an approach based on two-photon excitation imaging to quantify insulin exocytosis in intact pancreatic islets (Takahashi N *et al. Science* **297**, 1349, 2002). This approach has been designated TEP (two-photon extracellular polar-tracer) imaging and TEPIQ (TEP imaging-based quantification) analysis (Kasai H. *et al. J. Physiol.* **568**, 891, 2005). TEP imaging is able to monitor reliably and with a relatively high time resolution (<1 s) individual insulin exocytic events in intact islet preparations and also allows analysis of the dynamics of the fusion pore that mediates exocytosis.

He first examined the participation of PKA in GIE with the use of TEP imaging, in which insulin exocytic events were visualized by two-photon imaging of islets immersed in an extracellular solution containing the polar fluorescent tracersulforhodamine B (SRB). He detected exocytic events as discrete spots of fluorescence, which reflected diffusion of SRB into individual insulin granules via the fusion pore. The intensities of the spots of SRB fluorescence were consistent with them reflecting exocytosis of large dense-core vesicles. Moreover, he could detect all the exocytic events in the region of interest by this approach, because the measured rate of GIE was similar to the value obtained for mouse islets by radioimmunoassay.

When he pretreated the cells with various inhibitors of PKA and then stimulated with 20 mM glucose, he found that the PKA inhibitors markedly and selectively inhibited the initial period (~250 s) of the first phase (< 7 min) of GIE. In contrast, forskolin, which increases the cytosolic concentration of cAMP by activating adenylyl cyclase, increased the extent of secretion both during the first phase of GIE and during the second phase, consistent with their previous observations. These results thus provided the notion that PKA is required for the initial period of the first phase of GIE.

He also measured the possible effects of PKA inhibitors and forskolin on glucose-induced increases in $[Ca^{2+}]_i$ in islets. Increases in $[Ca^{2+}]_i$ were measured with either the high-affinity Ca^{2+} indicator fura-2 (K_d = 0.18 μM) or the low-affinity Ca^{2+} indicator fura-4F (K_d = 1.16 μM); the latter was used in case fura-2 became saturated during the physiological increases in $[Ca^{2+}]_i$. He found that neither the onset nor the maximal value apparent within 250 s of the glucose-induced increases

in $[Ca^{2+}]_i$ was affected by PKA inhibitors.

Thus, he next examined whether glucose and PKAmight directly potentiate CIE. For these experiments, Ca^{2+}-dependent mechanisms were saturated by large increases in$[Ca^{2+}]_i$ generated by photolysis of the caged-Ca^{2+} compound *o*-nitrophenyl-EGTA (NPE). He confirmed that irradiation with UV light induced an abrupt increase in $[Ca^{2+}]_i$ of >20 μM. The latency histogram for the discrete exocytic events was fitted by a probability density function with two exponential components, consistent with the characteristics of CIE studied by amperometry.

When islets were exposed to a high glucose concentration (20 mM) for only 1 min before uncaging of NPE, during which time glucose alone did not increase $[Ca^{2+}]_i$, the extent of CIE was markedly increased. Also, the glucose action was not mimicked by 2-deoxy-D-glucose (20 mM), indicating that it required a metabolite of glucose. The effect of glucose on CIE was abolished by pretreatment of islets with inhibitors of PKA. Furthermore, forskolin did not significantly affect CIE at the low glucose concentration of 2.8 mMbut potentiated the effect of 20 mM glucose on CIE. These results suggested that cAMP is necessary but not sufficient for the rapid effect of glucose on CIE, and that a metabolite of glucose, such as ATP, is required for this actionof glucose.

Finally, he probed the dynamics of the exocytic fusion pore by simultaneous imaging of two fluorescent tracers with different molecular sizes, SRB (~1.4 nm) and 10-kDa fluorescein dextran (~6 nm). Transient opening of the initial small pore was detected in 6.5% of events. The frequency of such transient opening was reduced to 3.8% in the presence of forskolin and increased toabout 10% in the presence of PKA inhibitors. These results suggested that PKA affects the fusion pore when its diameter is < 6 nm, and that the action of PKA is mediated, at least in part, at the level of the fusion reaction.

In conclusion, with the use of TEP imaging, he has now shown that a PKA-dependent mechanism, operative at the basal level of PKA activity, is important for the initial period of the first phase of GIE in mouse pancreatic islets. Furthermore, he found that PKA mediates rapid enhancement of CIE in islets only in the presence of high glucose concentration, indicating that PKA plays a glucose sensing role, specifically in the first phase of GIE. Given that the first phase of GIE is reduced in many individuals with type 2 diabetes mellitus from an early stage of the disease, impairment of this mechanism may contribute to the pathogenesis of this condition

論文の審査結果の要旨

グルコースは、膵ランゲルハンス島β細胞からのインスリン分泌を強く促進する。グルコースは、in vivoのみならず単離膵島においても二相性のインスリン分泌を引き起こし、その作用にはK_{ATP}チャネルの閉鎖による細胞内カルシウム濃度の上昇が重要と考えられている。一方、単離膵島においてcAMP依存性プロテインキナーゼ（Protein kinase A, PKA）を活性化すると、グルコース刺激によるインスリン分泌を強く促進することが報告されているが、PKAの作用機構は不明であった。申請者は、本研究において、PKAがグルコースによるインスリン分泌顆粒の早期開口放出に重要な調節作用を営んでいるかを、二光子励起画像法を用いて明らかにした。申請者は、共同研究者としてこの測定法の開発にも参画し、マウス単離膵島における個々のインスリン分泌顆粒の開口放出を、秒単位でリアルタイムに観察・定量化することに成功、この測定法を用いることによってPKAの調節作用を明らかにした。

グルコース刺激によるインスリン開口放出にPKAがどのような調節作用を営んでいるかを明らかにするため、申請者はまず、単離したマウス膵島を用いてPKA阻害薬の効果を調べた。その結果、水溶性の高いPKA阻害薬が、グルコース刺激（20mM）後250秒以内に起こるインスリンの早期開口放出を選択的に抑制することを見いだした。同様に、脂溶性の高いPKA阻害薬もインスリンの早期開口放出を抑制することを、10数個程度の膵島細胞からなる少数の細胞塊を用いて確認した。さらに申請者は、アデニルシクラーゼを活性化するforskolinが、250秒以内に起こるインスリンの早期開口放出を逆に促進することを示した。

次に申請者は、グルコース刺激後の細胞内カルシウム濃度上昇に、PKAがどのような調節作用を営むかを調べた。その結果、PKA阻害薬とforskolinは、グルコースによるカルシウム濃度上昇に効果を及ぼさなかった。そこで申請者は、ケイジドカルシウム試薬を用いて20 μM以上のカルシウム濃度上昇を作りだし、これによって起こるインスリン分泌顆粒の開口放出に対してグルコース及びPKAがどのような調節作用を及ぼすかを調べた。その結果、20mM のグルコースを作用させ、1分後にケイジドカルシウム試薬によってカルシウム濃度を上昇させると、カルシウム濃度上昇には差を認めないにも関わらず、開口放出が2倍に増加することを見いだした。またPKA阻害薬はこのグルコース増強効果を完全に抑制し、forskolinはその効果を逆に増強した。一方、非代謝性グルコース誘導体（2-deoxyglucose, 20mM）ではこのような増強効果を認めなかった。以上の実験結果から、グルコースは、細胞内カルシウム濃度を上昇させるだけでなく、カルシウム濃度上昇後のインスリン開口放出にも関与することが明らかとなった。しかもPKAは、グルコース代謝との協同作用により、このカルシウム濃度上昇後のインスリン開口放出に促進作用を及ぼすことが判明した。

最後に申請者は、インスリン分泌顆粒膜と細胞膜との融合反応にPKAがどのような調節作用を営んでいるかを調べた。その結果、PKAが、インスリン分泌顆粒膜と細胞膜が融合して細孔を形成し開口放出に至る、細孔の初期開大過程を促進することを見いだした。

このように本研究結果は、インスリン分泌に及ぼすPKAの調節作用について重要な知見を提供するものである。本研究は、PKAの作用機構を分子レベルで解明するには至っていないものの、研究結果は二光子励起画像法を用いなければ発見できなかった新しい知見であり、当該分野における研究の進展に大きく貢献した。よって申請者の論文は学位論文として十分ふさわしい内容であると審査委員会の委員全員一致で判定した。