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Real Time Electrochemical Detection of 5-HT/Insulin Secretion from Single Pancreatic Islets: Effect of Glucose and K^+ Depolarization

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We report a highly sensitive electrochemical approach suitable for the real time measurement of insulin release from single islets of Langerhans, the functional endocrine units in the pancreas. The method is based on the detection of the insulin surrogate 5-hydroxytryptamine (5-HT) by carbon fibre microelectrodes implanted in the islets. Based on the combination of this novel approach with the simultaneous microfluorometric recording of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), we demonstrate that glucose-stimulated islets secrete 5-HT/insulin in a pulsatile fashion under physiological conditions, and that this activity is encoded by synchronous $[Ca^{2+}]_i$ oscillations. The sensitivity to detect variations in minute amounts of secreted materials is partially conferred by the fact that the tracer is released into a relatively confined space (the intraislet interstitial space).

Pancreatic islets (about 80% of which are J-cells) are functional units specialized in the release of insulin which follows a rise in blood glucose. It has long been known that individual islets secrete insulin in a pulsatile fashion when stimulated by the hexose (1-4). However, the available discrete procedures to detect the hormone (RIA and, more recently, ELISA) lack the time resolution required to appreciate the full dynamics of the secretory process. This, together with the fact that the islet tissue is scarce, makes it of paramount importance that alternative, real-time methods are devised to follow the time course of secretion at the single islet level.

When applied externally, the electroactive amine 5-HT enters J-cells, accumulates in acidic insulin granules and is co-released with the hormone in response to appropriate stimuli (5-7). Indeed, 5-HT has been recently used as an effective insulin tracer in microamperometric studies of individual J-cells (8,9). We report here, for the first time, a 5-HT-based electrochemical approach suitable for the real time detection of insulin secretion from single islets, and demonstrate that, in the presence of stimulatory glucose levels, 5-HT/insulin release is indeed pulsatile under physiological conditions. The method is applicable to the study of the action of metabolizable as well as non-metabolizable (eg. high K^+) secretagogues and is expected to be of general applicability to monitor *in situ* the release of minute amounts of hormones from different endocrine systems, provided that interstitial diffusion of the tracer is restricted and that it accumulates in the respective secretory granules.

MATERIALS AND METHODS

Collagenase-isolated mouse islets were cultured in RPMI 1640-based medium containing 11 mM glucose (first 5-7 h) and 5.5 mM glucose + 1 mM 5-HT (remaining 17-19 h). Islets were subsequently loaded with the fluorescent Ca^{2+} indicator fura-2 as described (10,11) and transferred to a perifusion chamber placed on the stage of an inverted fluorescence microscope, where they attached to the poly-L-lysine-coated glass bottom. Islets were perifused at 37°C with the following salt solution (mM): 120 NaCl, 5 KCl, 25 $NaHCO_3$, 2.56 $CaCl_2$, 1.1 $MgCl_2$ and glucose (gassed with 95% O_2/5% CO_2 for a pH of 7.4).

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The release of pre-loaded 5-HT was monitored amperometrically using glass-encased carbon fiber microelectrodes, made essentially as described (12). The protruding carbon fiber (8 Tm diameter) was bevelled at 30^0 on a micropipette grinder (EG-4, Narishige, Japan) and gently implanted in the islet. Amperometric currents were measured at a potential of +0.55 V vs Ag/AgCl, using a nano-amperometer (AMU 130, Tacussel/Radiometer, France; sensitivity range 0.1 pA-20 nA). Amperometric current was low-pass filtered (100 Hz), amplified 10x and permanently stored on a DAT recorder (DTR-1200, Biologic, France). The data were transferred off-line at 1 kHz to a computer via a D12-IEEE/N interface (Biologic) using in-house software (13), and digitally refiltered (FFT smoothing provided by Microcal Origin) in order to eliminate the contaminating 50 Hz noise. This smoothing procedure did not affect fast amperometric current transients (≈100 ms). We note that the amperometric current declined at a very high rate immediately after inserting the electrode in the islet; electrode stabilization was essentially reached in less than 15 min, but the current continued to decline at a very low rate thereafter.

The $[Ca^{2+}]_i$ was fluorometrically recorded simultaneously with the amperometric current, using a dual-excitation system (Deltascan, PTI, USA) as described (10,11). Effective synchronization between the fluorescence and amperometric recordings was achieved by applying simultaneous time marking events to both systems.

Fura-2/AM was from Molecular Probes. 5-HT hydrochloride and all other chemicals were from Sigma.

RESULTS AND DISCUSSION

Fig.1A (lower trace) shows a typical *in situ* amperometric recording of 5-HT using a carbon fibre microelectrode, obtained in the presence of 11 mM glucose. Since the islet diameter is 200-300 Tm (equivalent to 17-25 J-cell diameters) and the protruding carbon fibre tip is 30-50 Tm long, the microelectrode probes a restricted interstitial domain (henceforth designated intra-islet *pocket*) located above the respective equatorial plane (Fig. 1E). The amperometric current exhibited periodic fluctuations at a frequency of 2-5 min^{-1} (average 3.2 + 1.2 min^{-1}; + S.D., n = 12 islets), indicating that 5-HT was released to the intra-islet *pocket* in a pulsatile fashion.

We have simultaneously recorded cytosolic Ca^{2+} using the fluorescent indicator fura-2 and found that the $[Ca^{2+}]_i$ was also oscillatory (Fig. 1A, upper trace), as previously reported (14,15). Although the calcium signal is expected to originate mainly from cell layers within the islet lower hemisphere (shadowed area in Fig. 1E), the $[Ca^{2+}]_i$ oscillations are remarkably synchronous with the 5-HT fluctuations (Fig. 1A). This reinforces the view that native J-cells are tightly coupled (14,16,17) and strongly suggests that the observed calcium signal provides a valid representation of the signalling properties of the J-cells contributing to the 5-HT fluctuations. Pulsatile 5-HT/insulin release appears therefore to be driven by the oscillatory $[Ca^{2+}]_i$ activity and underlying bursting electrical activity, as previously suggested (1,2,14).

We found that the time course of the descending phase of each secretory transient (average time constant for the experiment depicted in Fig. 1A: 6.8 + 2.0 s, n = 10 oscillations) was similar to that of the exponential current decay that could be recorded *in situ* following delivery of a pulse of a 5-HT-containing solution (Fig. 1B; time constant 9.4 s). Analysis of a pool of similar experiments indicated that the average time constant for the descending phase of the secretory transients (5.8 + 2.1 s, n = 5 islets) was not significantly different from the time constant calculated from the bath applications of 5-HT (6.6 + 2.4 s). This suggests that the descending phase of the 5-HT transients might be controlled by 5-HT washout from the intra-islet *pocket*, rather than by the decaying phase of the associated $[Ca^{2+}]_i$ rise. The $[Ca^{2+}]_i$ remains high throughout action potential firing and starts decaying as soon as the latter ceases, both in bursting islets (14) and single firing J-cells (18). However, exocytosis is specifically supported by the active phase (18), in agreement with our hypothesis.

When monitored by cell capacitance, exocytotic release in J-cells is virtually instantaneous with respect to the triggering calcium signal (18,19). In contrast, the rising phases of the intra-islet 5-HT transients have a slower time course than the corresponding $[Ca^{2+}]_i$ rises. Indeed, for the experiment illustrated in Fig.1A the average half-rising times of the $[Ca^{2+}]_i$ and amperometric current transients were 1.9 and 3.1 s, respectively, corresponding to a lag time of 1.2 s (lag time range, determined from other experiments: 0.3-3 s). Response delays may be

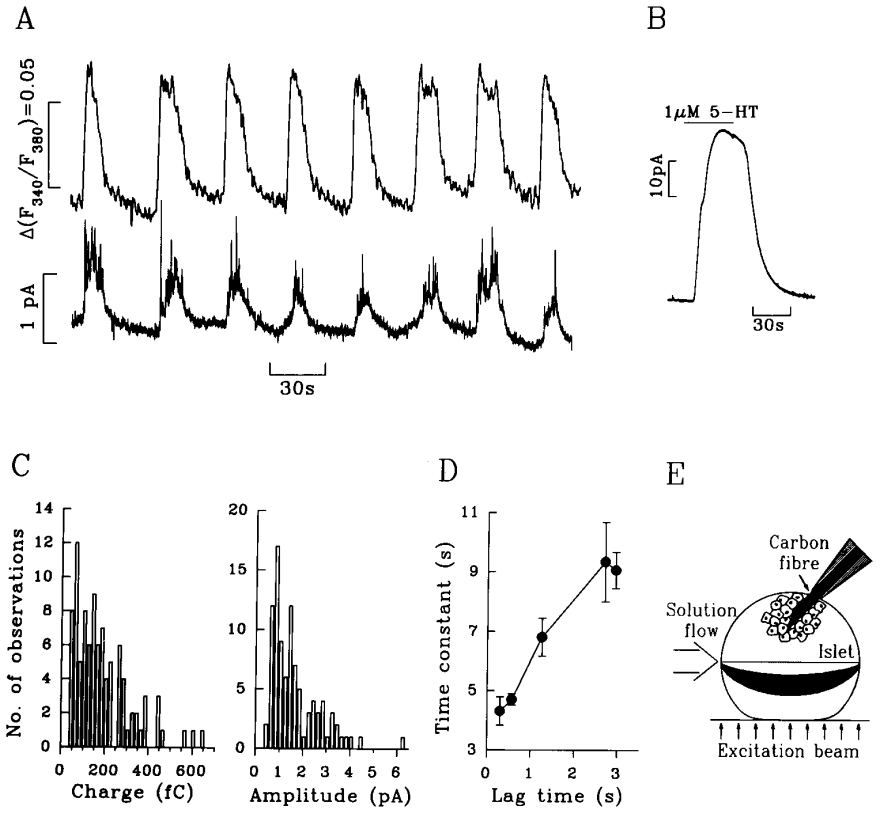

FIG. 1. Oscillatory $[Ca^{2+}]_i$ and 5-HT release, recorded simultaneously from single pancreatic islets. (A) Upper trace: continuous microfluorometric recording of $[Ca^{2+}]_i$ from a single fura-2/5-HT-loaded islet, as given by the ratio of fura-2 fluorescence at 340 and 380 nm (F_{340}/F_{380}). Lower trace: continuous recording of amperometric current from the same islet through a carbon fibre microelectrode. Glucose concentration was 11 mM throughout. (B) Effect of applying a step pulse (bar) of a 5-HT-containing solution on the amperometric current recorded *in situ.* (C) Amplitude histograms for the integral charge and peak amplitude of fast amperometric current transients. Calculated mean values: 188 ± 131 fC (median 156 fC) and 1.6 ± 1.0 pA (median 1.4 pA). Data (n = 95 spikes) from 2 islets displaying active spiking in 11 mM glucose. (D) Relationship between the time constant for 5-HT washout from the intra-islet interstitial space (calculated from the exponentially declining phases of the 5-HT transients) and the lag time between the $[Ca^{2+}]_i$ and the associated 5-HT transients (difference between the respective half-rising times). The time constants are plotted as the mean value ± S.E.M. for 10 oscillations (11 mM glucose, 5 different islets). (E) Schematic representation of the islet, highlighting the implanted microelectrode and the intra-islet *pocket* yielding the amperometric signal. Although we have routinely focused the fluorescence on the islet outermost rim (line), the signal is expected to originate mainly from the islet lower hemisphere (shadowed area), owing to non-confocality, excitation attenuation by the tissue and fluorescence self-absorption (14).

accounted for by a model whereby diffusion of 5-HT released into the intra-islet *pocket* is the rate limiting step for its accumulation. Taking the observed lag time (t) as the average diffusional lapsed time, the average distance of diffusion ($\langle x \rangle$, assumed to be a suitable estimate for the average *pocket* radius) is given by $\langle x \rangle = 2(Dt/X)^{1/2}$, where D is the 5-HT diffusion coefficient. Thus, for the experiment depicted in Fig. 1A the average *pocket* volume can be estimated to be equivalent to 110 J-cell volumes (assuming $D = 5.4 \times 10^{-10}$ $m^2.s^{-1}$ (20) and a J-cell radius of 6 Tm; *pocket* volume range, estimated from other experiments: 14-430 J-cell volumes). Dissipation of accumulated 5-HT would be expected to become slower as the pocket volume rises (i.e. as the lag time increases), as observed (Fig. 1D).

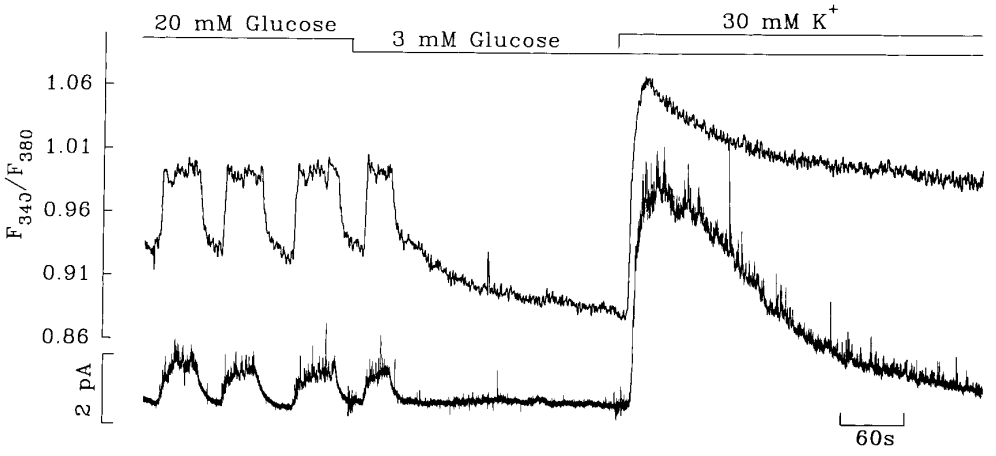

FIG. 2. High K^+-evoked 5-HT release and underlying $[Ca^{2+}]_i$ rises, recorded simultaneously from a single islet. Upper trace: continuous microfluorometric recording of $[Ca^{2+}]_i$ from a single fura-2/5-HT-loaded islet, as given by the ratio of fura-2 fluorescence at 340 and 380 nm (F_{340}/F_{380}). Lower trace: continuous recording of amperometric current from the same islet through a carbon fibre microelectrode. Glucose concentration was lowered from 20 to 3 mM and the islet subsequently exposed to 30 mM KCl, as shown.

Also significantly, fast current spikes were frequently seen in the amperometric recordings, most often associated with the rising phase and peak of the 5-HT transients (Fig. 1A). The frequency distributions of the respective integral charge and peak amplitude (Fig. 1C) bear a striking similarity to those reported for single stimulated J-cells (8), indicating that the observed spikes represent the contribution of discrete secretion events from individual J-cells lying in close contact with the microelectrode. According to our diffusional model, quantal 5-HT released from J-cells located further away from the electrode must spread out across wider distances, thus originating the smoother portions of the rising phases of the 5-HT transients.

Fig. 2 shows that our electrochemical detection system can probe 5-HT/insulin secretion evoked by metabolizable as well as non-metabolizable stimuli. Stimulation with high K^+ is well known to depolarize the J-cell membrane (21), leading to the activation of voltage-sensitive Ca^{2+} channels and to sharp $[Ca^{2+}]_i$ rises (10), a phenomenon that we have also observed in the presence of sub-stimulatory glucose levels (Fig. 2, upper trace, right). Significantly, high K^+ stimulation triggered a massive 5-HT/insulin release, the time course of which was identical to that of the concomitant $[Ca^{2+}]_i$ rise at the beginning of stimulation. However, at a later stage the $[Ca^{2+}]_i$ remained high whereas the amperometric current decayed to near-basal levels along a time course of minutes. While this apparent dissociation may reflect limited depletion of a ready-to-release pool of insulin granules and/or inactivation of voltage-sensitive Ca^{2+} channels, it is noteworthy that the 5-HT release rate is still quite high several minutes after beginning of stimulation, as can be inferred from the relative values of the decay time constants calculated in high K^+ (180 s) and throughout the descending phase of the 5-HT transients in 20 mM glucose (7.5 s; a measure of the rate of 5-HT washout from the intra-islet *pocket*, see above).

In summary, we report a novel approach for the real-time assessment of insulin release from single pancreatic islets. Previous studies (1,2), based on discrete sampling methods to detect insulin release, generally required islet exposure to high glucose and extracellular Ca^{2+} concentrations (15-20 and 8-10 mM, respectively) to enhance islet output and inter-burst separation, thus making pulsatile release observable. The enhanced sensitivity and time resolution of our method, however, enabled the observation of pulsatile insulin release under more physiological

conditions. Owing to the extended syncythial properties of the islets, the present method can also be used to relate the time course of the insulin fluctuations to that of the triggering signals ($[Ca^{2+}]_i$ rises), although some care should be taken to account for intra-islet diffusional delays and for the inherent lag times between both signals. The existence of relatively tight diffusional *pockets* in the islet, however, may turn out to represent a surendipitous bonus, offering enhanced sensitivity to detect minute amounts of secreted materials.

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