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Protein kinase C isoform specificity of cholinergic potentiation of glucose-induced pulsatile 5-HT/ insulin release from mouse pancreatic islets

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ABSTRACT

Thymeleatoxin (TMX), an activator of Ca2+-sensitive protein kinase C (cPKC) isoforms, was used to assess the PKC isoform specificity of cholinergic potentiation of glucose (11 mM)-induced pulsatile 5-HT/insulin release (PIR) from single mouse pancreatic islets. TMX (100 nM) and carbachol (Cch, 50 μM) enhanced PIR ~ 3-fold while reducing the underlying [Ca2+]i oscillations (duration and amplitude) by ~ 40-50%. Both effects were ablated by the specific PKC inhibitor bisindolylmaleimide and chronic TMX pretreatment. Cch also evoked an initial transient [Ca2+]i rise and surge of 5-HT release, which remained unaffected by chronic TMX pretreatment. It is concluded that the immediate cholinergic responses are insensitive to cPKC. In contrast, specific activation of a cPKC isoform mediates sustained cholinergic potentiation of glucose-induced insulin secretion.

Key terms: Cytosolic free Ca^{2+} concentration, 5-HT amperometry, islet of Langerhans, protein kinase C, pulsatile insulin release, thymeleatoxin.

PROLOGUE

This paper is presented in homage to Eduardo Rojas. He was the Ph.D. supervisor of two of us (RMS and LMR), while chairing the Biophysics Department of the School of Biological Sciences, University of East Anglia, UK. We were deeply touched by his vivacious and enthusiastic approach to research, which made the day-a-day life in the laboratory look like a never-ending intellectual and emotional challenge. He always enjoyed developing new techniques and new approaches to problems, thus inspiring his collaborators to do the same. We believe that the techniques used in this paper are an example of this attitude. Guayo was also special for his cheerfulness, and he remains a very special friend.

INTRODUCTION

Although glucose is the primary physiological trigger and regulator of insulin release, this is also amenable to modulation by the parasympathetic nervous system, which relies mostly on acetylcholine release from islet nerve endings and its binding to specific β-cell muscarinic (M_3) receptors (Prentki and Matschinsky, 1987; Verspohl et al., 1990). Cholinergic stimulation of pancreatic β-cells involves phosphoinositide-specific phospholipase C (PLC) activation and the consequent generation of both inositol 1,4,5-trisphosphate (1,4,5-IP_3), which triggers Ca^{2+} release from internal stores, and 1,2-sn-diacylglycerol (DAG) (Nishizuka, 1986; Wollheim and Biden, 1986), which may lead to the activation of

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either conventional (i.e., Ca^{2+}- and DAG/phorbol ester-sensitive) or novel (i.e., Ca^{2+}-insensitive and DAG/phorbol ester-sensitive) protein kinase C (PKC) isoforms (cPKC and nPKC, respectively).

The available evidence, largely based on studies involving rat islet β-cells and insulin-secreting cell lines, indicates that PKC activation is essential for sustained cholinergic regulation of glucose-induced insulin release (Hughes et al., 1990; Persaud et al., 1991; Tian et al., 1996; for review see Gilon and Henquin, 2001). The nature of the PKC isoforms involved is contradictory. Studies involving β-cell lines show that, while in RINm5F cells the cholinergic response is mediated by an atypical isoform (aPKC, i.e., Ca^{2+}- and DAG/phorbol ester-insensitive) (Tang and Sharp, 1998), in MIN6 cells, the contributing isoforms are phorbol ester-sensitive (i.e., cPKC and/or nPKC) (Pinton et al., 2002; Tian et al., 1996). Studies using whole rat islets indicate the involvement of nPKC isoforms (Harris et al., 1996; Ishikawa et al., 2005). No information is yet available in this respect for mouse islet β-cells which, contrary to rat islet β-cells (Antunes et al., 2000), exhibit a pattern of pulsatile insulin release related to bursting electrical activity and corresponding $[Ca^{2+}]_i$ oscillations (Atwater et al., 1978; Barbosa et al., 1998).

Thymeleatoxin (TMX) is a mezerein analogue that has been reported to act as a specific activator of cPKC isoforms, inasmuch as it does not activate Ca^{2+}-insensitive PKC isoforms for concentrations up to ~ 1.6 μM (Ryves et al., 1991). Accordingly, the toxin has been used to assess the differential role of cPKC and non-cPKC isoforms in different cell types (Llosas et al., 1996; Terzian et al., 1996; Yanagita et al., 2000). In this study, we have investigated the modulation of pulsatile insulin release and the underlying oscillatory Ca^{2+} signaling by TMX. We focused, in particular, on the role of TMX-sensitive PKC in cholinergic regulation of insulin secretion from mouse islets, in order to find evidence for PKC isoform specificity.

MATERIALS AND METHODS

Islet preparation and handling

Mouse islets were isolated by collagenase digestion and maintained in culture for 24-48 h prior to the experiments, as reported (Silva et al., 1994). Islets were cultured in RPMI 1640-based medium containing 11 mM glucose (first 5-7 h) and 5.5 mM glucose + 1 mM 5-hydroxytryptamine (5-HT, remaining time). Islet loading with the fluorescent Ca^{2+} indicator fura-2 (exposure to its acetoxymethyl ester fura-2/AM at 4 μM for 45 min) was carried out as described (Salgado et al., 1996; Silva et al., 1994). Fura-2/5-HT-loaded islets were transferred to a fast perifusion chamber placed on the stage of an inverted epifluorescence microscope. Stable islet attachment to the poly-L-lysine-coated glass chamber was achieved in less than 15 min under stationary conditions. Islets were then perifused with the following salt solution (in mM): 125 NaCl, 5 KCl, 25 $NaHCO_3$, 2.56 $CaCl_2$, 1.1 $MgCl_2$ and 11 glucose, constantly gassed with 95% O_2/5% CO_2 for a pH of 7.4, at a flow rate of 1.5-2 mL/min. Temperature in the chamber was 37ºC.

Microfluorometry

Cytosolic free Ca2+ concentration ([Ca2+]i) was recorded from single islets using a dual-excitation microfluorescence system, as reported (Salgado et al., 1996; Silva et al., 1994). Briefly, fura-2 was excited at 340 and 380 nm via two monochromators, and the fluorescence detected by a photomultiplier after passing through a band-pass interference filter centered at 510 nm. The data were automatically corrected for background fluorescence and acquired at 10 Hz by a computer. The fluorescence ratio F340/F380 was converted into [Ca2+]i values, as previously described (Salgado et al., 1996; Silva et al., 1994).

Microamperometry

The release of preloaded 5-HT was monitored amperometrically using glass-encased carbon fiber microelectrodes, as

reported for single cells (Smith et al., 1995; Zhou and Misler, 1996) and single islets (Barbosa et al., 1996; Barbosa et al., 1998). Briefly, the protruding carbon fiber tip (~ 8 μm diameter) of the microelectrodes was fully implanted in the islet tissue. The currents were measured using a picoamperometer (maximum sensitivity 0.1 pA) with the working electrode held at a potential of +0.55 V vs. Ag/AgCl. The current was low-pass filtered, amplified 10x and permanently stored on digital tape. The data were transferred off-line at 1 KHz to a computer where they were subsequently refiltered via an FFT smoothing routine provided by commercially available software (Microcal Origin), in order to eliminate 50 Hz mains interference.

Materials

Fura-2/AM was from Molecular Probes (Eugene, OR, USA). TMX was from Calbiochem (San Diego, CA, USA). Bisindolylmaleimide I (GF 109203X) was from LC Laboratories. All other chemicals were from either Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany). TMX and bisindolylmaleimide were prepared in DMSO, sampled in small aliquots, and kept at -20°C prior to use.

Data analysis

Total charge per oscillation was determined as the time integral of the current after subtraction of the respective background area, as reported (Barbosa et al., 1998). Only high-frequency oscillations were considered for these calculations. Statistical significance of differences between mean values was assessed by paired Student's t-test. These differences were considered to be statistically significant at the 95% confidence level ($P<0.05$).

RESULTS

We have investigated the effects of TMX on glucose-induced $[Ca^{2+}]_i$ and 5-HT/insulin oscillations, recorded from whole mouse islets. Figure 1A (upper trace) shows that 11 mM glucose elicited a pattern of high-frequency $[Ca^{2+}]_i$ oscillations, as previously acknowledged (Barbosa et al., 1998; Santos et al., 1991). Exposing the islet to 100 nM TMX in the continued presence of glucose resulted in a marked increase in the frequency of the $[Ca^{2+}]_i$ oscillations from ca. 2.4 to 5.0 min^{-1}, ~ 20 min after toxin addition (average increase 71 ± 12 %; $P<0.05$, n=7 islets). The toxin also caused a reduction in both the duration and amplitude of these oscillations (see Fig.1B for the experiment depicted in A) which, on average, amounted to 23 ± 7% and 46 ± 8%, respectively ($P<0.05$, n=7 islets).

Also shown in Figure 1A (lower trace) is a simultaneous amperometric recording of 5-HT/insulin release. Current fluctuations were typically observed in presence of 11 mM glucose, as previously reported (Barbosa et al., 1996; Barbosa et al., 1998). These fluctuations arise from oscillatory release of 5-HT into the islet interstitial space probed by the microelectrode tip (Barbosa et al., 1996). We have assessed the amount of 5-HT released per oscillation into this space by determining the current integral for each cycle. TMX addition evoked a 1.5-fold increase in the charge released per oscillation (Fig. 1B), which on average corresponded to a 107 ± 15% increase ($P<0.005$, n=7 islets). Considering the TMX-evoked increase in oscillation frequency, the overall 5-HT release rate (estimated by multiplying the charge released per oscillation by the corresponding oscillatory frequency) increased by 316 ± 63% ($P<0.005$) (208% for the experiment depicted in Fig. 1A). The above parameters were all measured at the steady state in 11 mM glucose or 11 mM glucose + 100 nM TMX (i.e., after 20 min exposures to TMX).

Figure 2 depicts the effects of TMX on $[Ca^{2+}]_i$ and 5-HT oscillations in the continued presence of 11 mM glucose and 10 μM bisindolylmaleimide I, a specific broad-range PKC inhibitor (Martiny-Baron et al., 1993; Tang and Sharp, 1998), which did not appear to affect by itself the islet oscillatory activity. In this particular experiment, an irregular activity was observed, with slow oscillations underlying a pattern of high-frequency oscillations

both in control and in presence of TMX; this is a pattern that is seen occasionally in islets under physiological conditions and, therefore, unrelated to the presence of bisindolylmaleimide. Bisindolylmaleimide impaired the effect of TMX on both oscillatory frequency (5 ± 1 vs. 4.3 ± 0.8 min^{-1} in control, i.e., 11 mM glucose + bisindolylmaleimide; P>0.05, n=3 islets), overall 5-HT release rate (30 ± 11 vs. 24 ± 11 pC/min in control, P>0.05) and $[Ca^{2+}]_i$ oscillation responsiveness (oscillation amplitude: 48 ± 8 vs. 61 ± 19 nM in control; oscillation duration: 3 ± 1 vs. 4 ± 1 s in control, P>0.05).

Chronic TMX pretreatment is known to cause down-regulation of cPKC activity (Yanagita et al., 2000). We have investigated the effects of carbachol (Cch) on glucose-induced $[Ca^{2+}]_i$ and 5-HT/insulin oscillations, as well as the effect of chronic TMX pretreatment (100 nM, 20-24 h) on islet responsiveness to the muscarinic agonist, in order to find evidence for PKC isoform specificity of cholinergic regulation. As shown in Figure 3A, exposing the islets to 50 μM Cch in the continued presence of glucose elicited a rapid transient $[Ca^{2+}]_i$ rise, which was followed by the reappearance of the $[Ca^{2+}]_i$ oscillations. The frequency of the

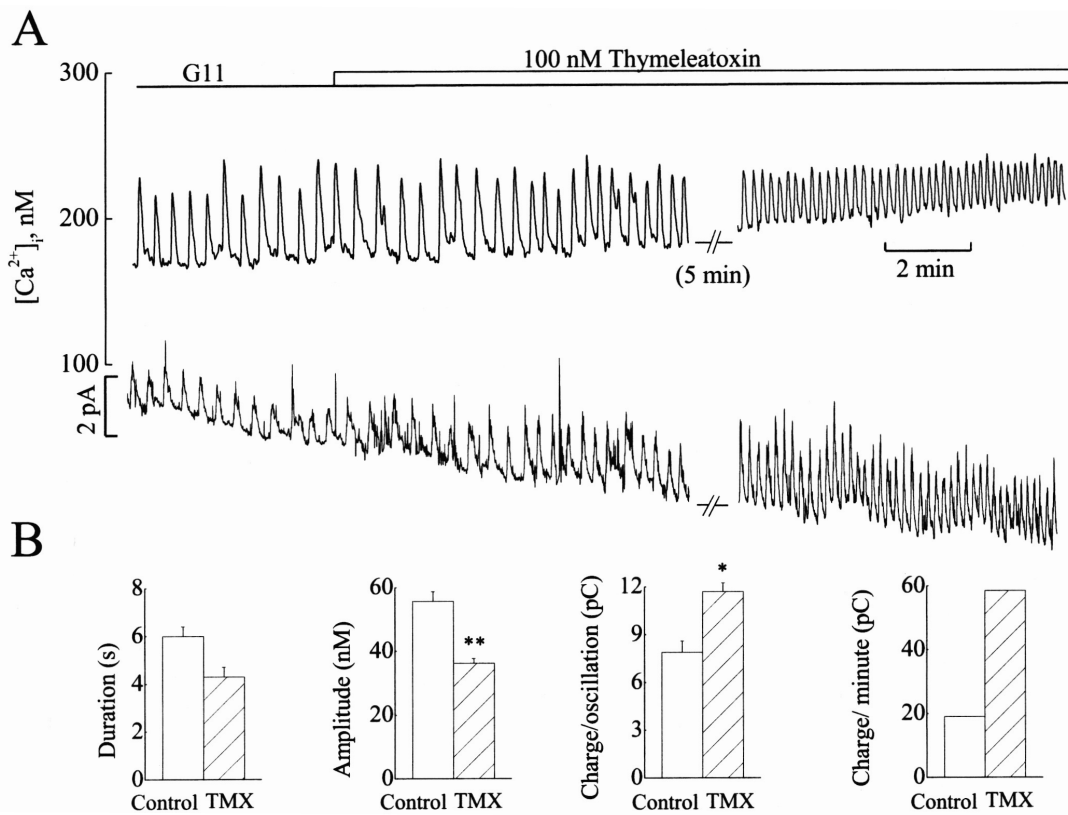

Figure 1. Effects of thymeleatoxin on glucose-induced $[Ca^{2+}]$ oscillations and pulsatile 5-HT release. **A:** Simultaneous recording of $[Ca^{2+}]_i$ (upper trace) and 5-HT/insulin release (lower trace) from a single fura-2/5-HT-loaded islet. The islet was exposed to 100 nM TMX in the continued presence of 11 mM glucose. **B:** Analysis of the effect of TMX on $[Ca^{2+}]_i$ oscillations (first two panels) in terms of oscillation duration (in s, first panel) and amplitude (in nM, second panel), as well as on 5-HT fluctuations (last two panels) in terms of time integral for each cycle (in pC, third panel) and overall 5-HT release rate (in pC/min, fourth panel). Data from the experiment depicted in A. All parameters were assessed 20 min after toxin addition to the perifusion solution, either in 11 mM glucose ("control") or in 11 mM glucose + 100 nM TMX ("TMX"). Data are presented as mean ± SEM (n=6 oscillations). *: P < 0.05; **: P<0.005.

$[Ca^{2+}]_i$ oscillations at the steady-state of Cch (i.e., ~ 20 min after its addition) was higher than in control (ca. 8.8 vs. 5.5 min^{-1}; average increase: 155 ± 12 %; P<0.0001, n=10 islets). Also significantly and similar to acute exposure to TMX, Cch caused a reduction in $[Ca^{2+}]_i$ oscillation duration and amplitude (Fig.3B). On average, Cch reduced $[Ca^{2+}]_i$ oscillation amplitude and duration at the steady state by 49 ± 6 % and 52 ± 7 %, respectively (P<0.05, n=10 islets).

The typical effect of Cch on glucose-induced pulsatile 5-HT/insulin release, as monitored by 5-HT microamperometry simultaneously with $[Ca^{2+}]_i$, is depicted in Figure 3A. Similar to the effect on $[Ca^{2+}]_i$, adding Cch elicited a pronounced transient rise in the current, which was followed by the reappearance of the 5-HT oscillations. Cch evoked a 3.5-fold increase in the charge released per oscillation at the steady-state (Fig. 3B) which, on average, amounted to a 53 ± 12% increase (P<0.05, n=10 islets). Considering the Cch-evoked increase in oscillation frequency, the overall 5-HT release rate increased by 225 ± 46% (P<0.005) (445% for the experiment depicted in Fig.3A). The above parameters were all measured at the steady state in 11 mM glucose or 11 mM glucose + 50 μM Cch (i.e., after 20 min exposures to Cch). The steady-state effects of Cch on pulsatile 5-HT/insulin release thus were similar to the effects of 20 min exposures to TMX.

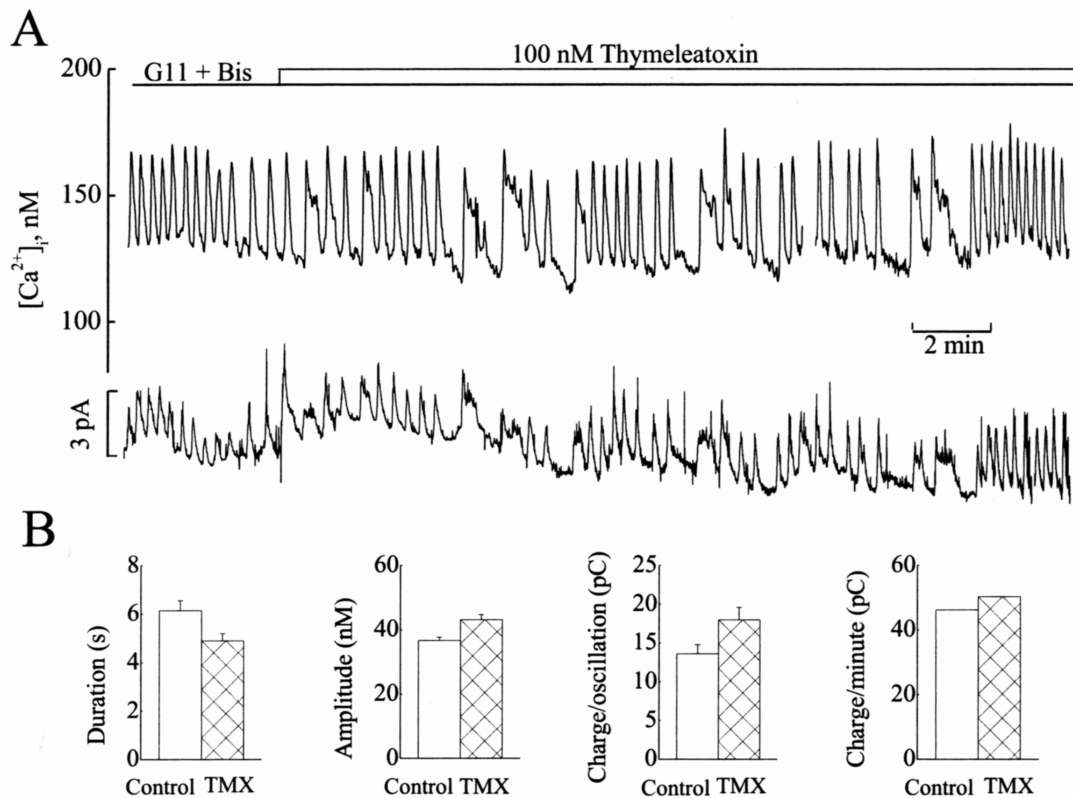

Figure 2. Reversal by bisindolylmaleimide of the effects of thymeleatoxin on glucose-induced $[Ca^{2+}]$ oscillations and pulsatile 5-HT release. **A:** Simultaneous recording of $[Ca^{2+}]_i$ (upper trace) and 5-HT/insulin release (lower trace) from a single fura-2/5-HT-loaded islet. The islet was incubated with 10 μM bisindolylmaleimide ("Bis") prior to (10 min) and during exposure to TMX (11 mM glucose throughout). **B:** Analysis of the effect of TMX on $[Ca^{2+}]_i$ (first two panels) and 5-HT oscillations (last two panels) (see legend to Fig.1B). Data from the experiment depicted in A. All parameters were assessed 20 min after toxin addition to the perifusion solution, either in 11 mM glucose + 10 μM bisindolylmaleimide ("control") or in 11 mM glucose + 10 μM bisindolylmaleimide + 100 nM TMX ("TMX"). Data are presented as mean ± SEM (n=6 oscillations).

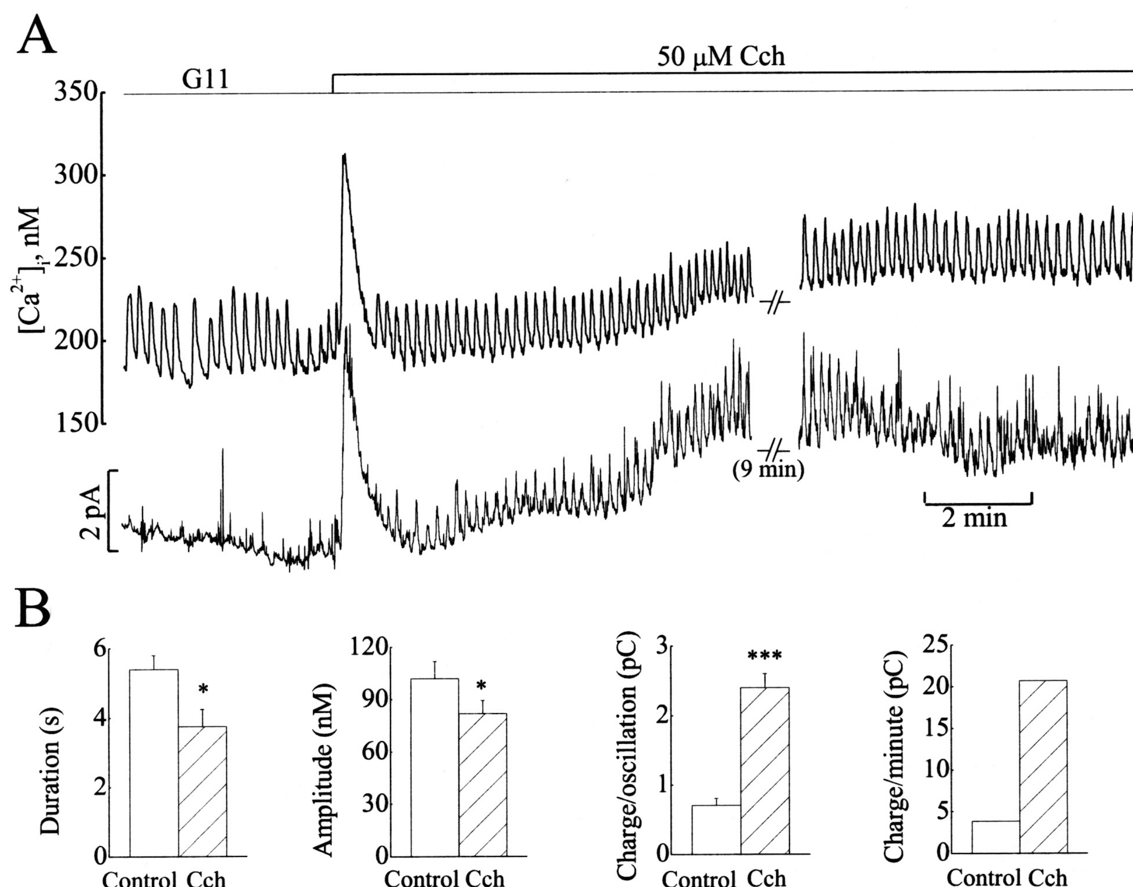

Figure 3. Effects of carbachol on glucose-induced $[Ca^{2+}]$ oscillations and pulsatile 5-HT release. **A:** Simultaneous recording of $[Ca^{2+}]_i$ (upper trace) and 5-HT/insulin release (lower trace) from a single fura-2/5-HT-loaded islet. The islet was exposed to 50 μM Cch in the continued presence of 11 mM glucose. **B:** Analysis of the effect of Cch on $[Ca^{2+}]_i$ oscillations (first two panels) and 5-HT oscillations (last two panels) (see legend to Fig. 1B). Data from the experiment depicted in A. All parameters were assessed at the steady-state of 11 mM glucose ("control") and 11 mM glucose + 50 μM Cch (i.e., 20 min after Cch addition; "Cch"). Data are presented as mean ± SEM (n=6 oscillations). *: P<0.05; ***: P<0.0001.

Figure 4 depicts the effects of Cch on $[Ca^{2+}]_i$ and 5-HT oscillations, recorded from an islet subjected to chronic TMX pretreatment (100 nM, 20 h). TMX-pretreated islets tended to exhibit shortened $[Ca^{2+}]_i$ oscillations, a decreased charge released per oscillation, and an enhanced oscillatory frequency. TMX pretreatment did not affect either the Cch-evoked initial transient $[Ca^{2+}]_i$ rise (130 ± 29 nM vs. 148 ± 19 nM for control, i.e., TMX-untreated islets) or the corresponding integrated 5-HT response (90 ± 24 pC vs. 121 ± 28 pC for control; P>0.05, n=9 islets). It did, however, impair the steady-state effect of Cch (Fig. 4B). Indeed, the average charge released per oscillation in Cch was 4 ± 1 vs. 3.4 ± 0.6 pC in control (P>0.05), and the overall 5-HT release rate was 16 ± 4 vs. 14 ± 3 pC/min in control (P>0.05). Moreover, average changes in $[Ca^{2+}]_i$ oscillation amplitude and duration relative to control were 25 ± 7 and 31 ± 18%, respectively (P>0.05, n=9 islets). The Cch-induced increase in oscillatory frequency also was not observed in TMX-pretreated islets (it actually decreased from 8 ± 1 in 11 mM glucose to 5 ± 1 min^{-1} in Cch; P<0.05, n=9 islets).

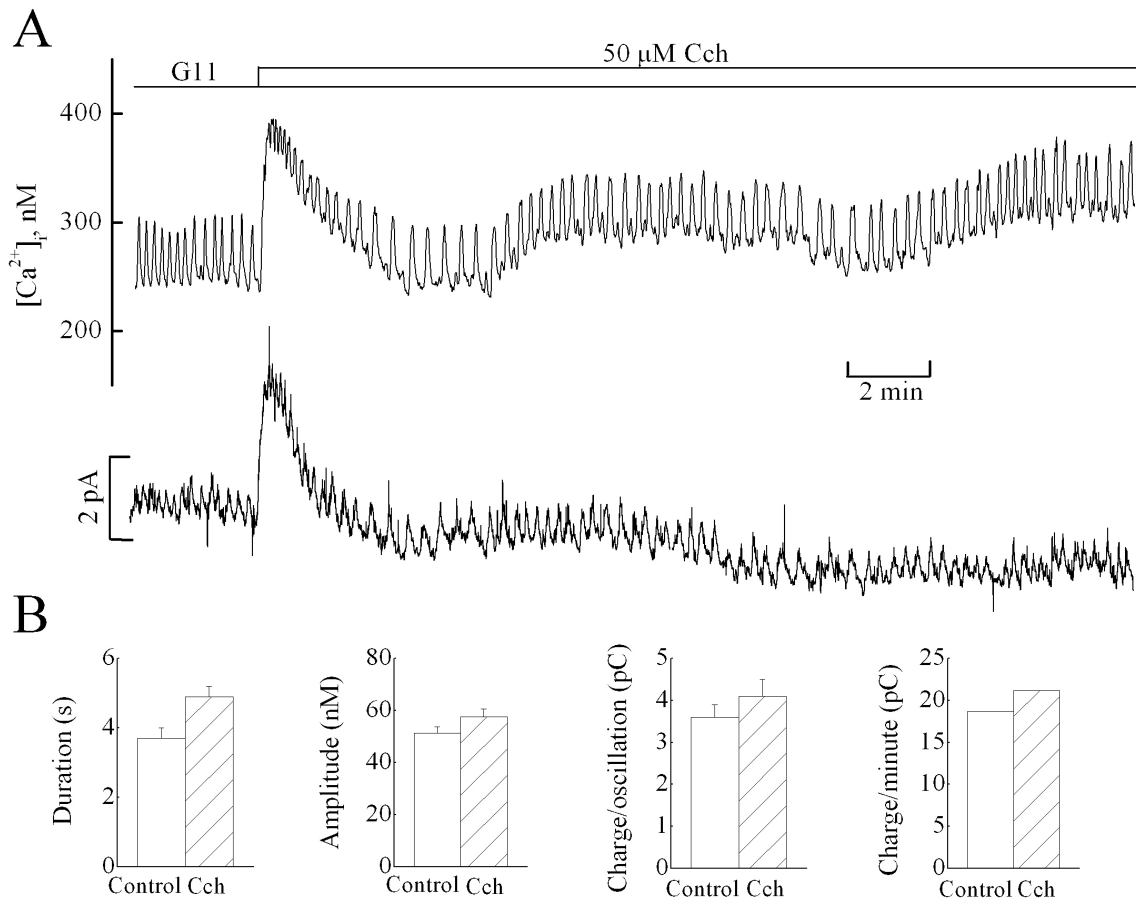

Figure 4. Reversal by chronic thymeleatoxin pretreatment of the effects of carbachol on glucose-induced [Ca2+] oscillations and pulsatile 5-HT release. **A:** Simultaneous recording of [Ca2+]i (upper trace) and 5-HT/insulin release (lower trace) from a single fura-2/5-HT-loaded islet. The islet was subjected to long-term (20 h) pretreatment with 100 nM TMX and left in toxin-free solution for 120 min prior to Cch addition. **B:** Analysis of the effect of Cch on [Ca2+]i oscillations (first two panels) and 5-HT oscillations (last two panels) (see legend to Fig.1B). The analysis referred to the experiment depicted in A. All parameters were assessed at the steady state of 11 mM glucose ("control") and 11 mM glucose + 50 μM Cch (i.e., 20 min after Cch addition; "Cch"). Data are presented as mean ± SEM (n=6 oscillations).

DISCUSSION

Using fast 5-HT microamperometry and fura-2 microfluorescence techniques to monitor pulsatile 5-HT/insulin release and $[Ca^{2+}]_i$ in real time from single glucose-stimulated mouse islets, we have shown that 100 nM thymeleatoxin reduces both the amplitude and duration of the $[Ca^{2+}]_i$ oscillations, while concomitantly enhancing pulsatile insulin release. It also augments the frequency of $[Ca^{2+}]_i$ and 5-HT oscillations. All these actions are ablated by treatment with the specific PKC inhibitor bisindolylmaleimide, indicating that they are mediated by PKC activation. Since, at the concentration used, TMX reportedly activates cPKC isoforms only (Ryves et al., 1991), the PKC isoform involved in toxin action is likely to be either PKC-α, PKC-β or both. Indeed, rat pancreatic β-cells and insulinoma cell lines express the cPKC isoforms α and β (Knutson and Hoenig, 1994; Tang and Sharp, 1998; Tian et al., 1996). We are investigating currently whether mouse β-cells express the same cPKC isoforms.

We also have shown that cholinergic stimulation elicits a multiple response

consisting of an initial transient $[Ca^{2+}]_i$ rise and concomitant surge of 5-HT/insulin release, followed by sustained $[Ca^{2+}]_i$ oscillations from a plateau. These oscillations had a reduced duration and amplitude and were accompanied by a marked potentiation of pulsatile 5-HT/insulin release. The overall cholinergic response resembles that observed in terms of electrical activity recorded from whole mouse islets, where acetylcholine evoked a membrane depolarization with an initial phase of continuous spiking followed by bursts of electrical activity with a lower duration and higher frequency (Hermans et al., 1987; Santos and Rojas, 1989). The initial response is likely to reflect Ca^{2+} release from internal stores via the PLC/1,4,5-IP_3 system (Gilon and Henquin, 2001; Hermans and Henquin, 1989). Significantly, this response remained unaffected by chronic TMX pretreatment (a procedure known to cause down-regulation of cPKC isoforms (Yanagita et al., 2000) and was not present when the islets were subjected to acute PKC stimulation with TMX. With respect to the secondary sustained response, it may reflect primarily the stimulation of Ca^{2+} sequestering/extrusion mechanisms (Arkhammar et al., 1989), as PKC activation reportedly enhances voltage-sensitive Ca^{2+} currents in b-cells (Ämmälä et al., 1994; Rorsman et al., 1986), and the activation of steps leading to the enlargement of the readily-releasable pool of insulin granules (e.g., granule translocation to the plasma membrane and/or priming of Ca^{2+}-sensitive proteins involved in granule fusion [Gillis et al., 1996; Rorsman and Renström, 2003; Vaughan et al., 1998]). Regardless of the ultimate nature of these mechanisms, the data presented strongly suggest that the sustained cholinergic response is mediated by the activation of a cPKC isoform. Indeed, this response is ablated by chronic thymeleatoxin pretreatment.

In conclusion, this study suggests that low concentrations of thymeleatoxin may be used to probe specific PKC isoforms in pancreatic β-cells. Activation of a cPKC isoform appears to mediate specifically the sustained stimulatory action of cholinergic agonists on pulsatile insulin release. This study calls for a close examination of the likely differential role of particular PKC isoforms in glucose- and receptor-response coupling in β-cells.

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