J. Membrane Biol. 148, 177–184 (1995)

The Journal of
Membrane
Biology

Ionic Mechanisms Involved in the Regulation of Insulin Secretion by Muscarinic Agonists

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Received: 21 March 1995/Revised: 26 July 1995

Abstract. The effects of the muscarinic agonist oxotremorine-m (oxo-m) on insulin secretion, K^+-permeability and electrical activity from isolated mouse pancreatic islets were studied. Oxo-m potentiated glucose-induced insulin secretion in a dose–dependent manner, saturating at ca. 10 μM. At 11.2 mM glucose, oxo-m (0.1 and 10 μM) had two distinct effects on β-cell electrical activity. Both concentrations increased the steady-state burst frequency, however, at 10 μM an initial and transient polarization was measured, and the subsequent activity was accompanied by a slight depolarization. The polarizing effect of oxo-m was almost completely suppressed by charybdotoxin (ChTX), a blocker of the large conductance (maxi) $[Ca^{2+}]_i$-activated potassium channel ($K_{(Ca)}$). In the presence of 11.2 mM glucose, oxo-m (50 μM) provoked a significant and transient increase in the ^{86}Rb efflux from perifused islets. This effect was inhibited by ChTX. ChTX also potentiated oxo-m stimulated insulin secretion in the presence of glucose. Finally, the balance between the polarizing and depolarizing effects of oxo-m was variable in different islets and depended on glucose concentration. Insulin secretion stimulated by oxo-m in the presence of glucose was more closely correlated to the agonist induced increase in burst frequency than to an increase in plateau fraction. We conclude that muscarinic stimulation has at least two effects on β-cell electrical activity, an initial hyperpolarization, owing to activation of $K_{(Ca)}$ channels, followed by depolarization and high-frequency bursts, proposed to reflect the activation of a current sensitive to the depletion of intracellular Ca^{2+} stores (CRAC).

Key words: Muscarinic agonist — β-cell electrical activity — Insulin secretion — K^+-permeability — CRAC — Charybdotoxin — Mouse

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Introduction

The cholinergic neurotransmitters are important modulatory factors in meal-related insulin secretion. Cholinergic stimulation increases insulin release through the activation of the muscarinic acetylcholine receptors (mAChR) present in β-cell membrane (Boschero et al., 1995). Muscarinic agonists promote hydrolysis of membrane phospholipids and generate IP_3, which mobilizes Ca^{2+} from intracellular stores (*see* Malaisse, 1986 for review). Radio-ligand binding analysis (Verspohl et al., 1990), functional studies (Henquin & Nenquin, 1988; Santos & Rojas, 1989) and c-DNA analysis (Boschero et al., 1995) have revealed that pancreatic β-cells possess the subtype M3 of the mAChR family. In agreement with these reports, there is evidence that neither changes in cyclic nucleotide concentrations (Gagerman et al., 1978) nor GTP-binding regulatory components of adenylate cyclase (Dunlop et al., 1988) are involved in the mAChR mediated activation of the β-cells.

In addition to the pharmacological and molecular characterization of muscarinic receptor subtypes in pancreatic islets, studies have shown that, in presence of glucose, acetylcholine (ACh) or carbachol, rapidly depolarizes the β-cell plasma membrane (Gagerman et al., 1978; Hermans, Schmeer & Henquin, 1987; Sanches-Andres, Ripoll & Soria, 1988). Furthermore, cholinergic effects on the electrical activity are qualitatively different from the effects of increasing glucose concentration (Cook, Crill & Porte, 1981). Practically every known ionic permeability has been postulated to explain the depolarization seen with cholinergic stimulation, e.g., decreased K^+ permeability (Santos & Rojas, 1989), in-

creased Na^+ permeability (Henquin et al., 1988; Hiriart & Ramirez-Medeles, 1993) and increased Ca^{2+} permeability (Sanchez-Andres et al., 1988).

Several agents have been used to characterize β-cell cholinergic responses in the past. Oxotremorine-m (oxo-m), a quaternary acetylenic amine, has been demonstrated to have the most favorable properties for studying muscarinic receptor activation in brain tissue (Birdsall, Burgen & Hulme, 1978; Sethy & Francis, 1990) and recently in islets of Langerhans (Boschero et al., 1995). Oxo-m has the added advantage over ACh of chemical stability and insensitivity to cholinesterases.

The main purpose of the present study was to re-evaluate the ionic mechanisms underlying the effects of muscarinic stimulation on the membrane electrical response in mouse pancreatic β-cells, using oxo-m as a new tool.

Materials and Methods

All experiments were performed on islets from the body and tail of the pancreas of adult swiss albino female mice, which were allowed free access to food and water, and were sacrificed by decapitation.

Electrophysiological Experiments

A piece of pancreas with a single partially dissected islet was fixed in a small chamber (40 µl vol) and perifused with a modified Krebs solution containing (in mM): 120 NaCl, 5 KCl, 25 $NaHCO_3$, 2.5 $CaCl_2$ and 1.1 $MgCl_2$. Solutions were continuously equilibrated with 95%O_2/5%CO_2 to maintain pH at 7.4 at 37°C. The membrane potential of a single β-cell was continuously recorded with a glass microelectrode (100–300 Mohms tip resistance). Cell input resistance was measured by intracellular current injection (−0.1 nAmp) through the same microelectrode used to measure membrane potential, as previously described (Atwater, Ribalet & Rojas, 1978). β-cells were identified by their characteristic burst pattern of electrical activity in the presence of 11.2 mM glucose. Electrophysiological records illustrate the typical responses of cells from at least 3 different islets.

Insulin Secretion Experiments

Islets were isolated aseptically by collagenase digestion. In brief, the pancreas was inflated with Hanks' balanced salt solution containing 3 mg collagenase/ml, excised and then continuously shaken at 37°C for 18 min. Isolated islets were then collected and cultured overnight in RPMI 1640 tissue culture medium (supplemented with 1% penicillin-streptomycin, 10% fetal bovine serum, 1% L-glutamine) at 37°C under an atmosphere of 95% air 5% CO_2. For insulin release measurements, batches of three islets each were preincubated at 37°C in 1 ml of modified Krebs solution enriched with 0.5% bovine serum albumin. The medium was then removed from each tube and replaced with fresh medium supplemented with glucose and/or oxo-m (six tubes of three islets each were exposed to each condition per experiment and each experiment was repeated at least twice). After 30 or 60 min incubation at 37°C, the supernatant was withdrawn for insulin measurements and the insulin content of the islets was extracted with 2 ml acid-ethanol. Insulin secretion was calculated as a percentage of total extracted insulin to adjust for the variable size of islets. The average insulin content per mouse islet was 390 ng in all experiments and experimental conditions, and ranged from 90 to 680 ng. The method used for insulin measurement was as previously described (Scott, Atwater & Rojas, 1981); the limit of detection is 2 pg insulin.

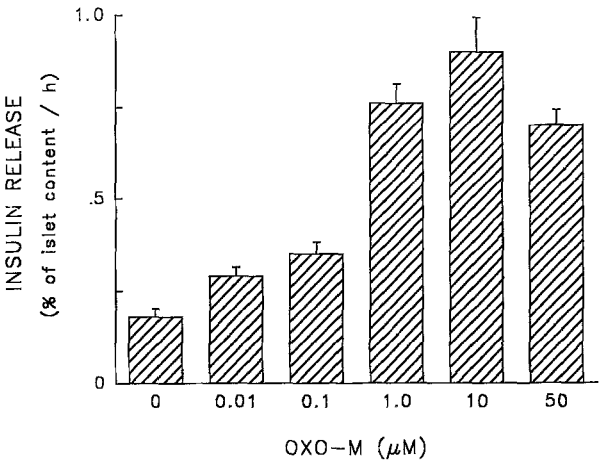

Fig. 1. Oxo-m stimulation of insulin release. Glucose (11.2 mM) was present throughout. Prior to the application of oxo-m, the groups of islets were preincubated for 45 min at 37°C. Next, the preincubation medium was replaced with Krebs solution containing increasing concentrations of oxo-m. Columns represent cumulative (60 min) insulin secretion as a percentage of total insulin content remaining in the islets, at the indicated concentration of oxo-m. Values are the Mean ± SE (n = 10–14 experiments). Insulin secretion expressed as ng per islet per 60 min for this experiment was: 0.60, 1.25, 1.64, 3.55, 3.00, and 2.98, respectively.

^{86}Rb Efflux

The method used to measure ^{86}Rb efflux has been described (Boschero & Malaisse, 1979). In this case, the ^{86}Rb efflux was expressed as fractional outflow rate (% of instantaneous islet content per 20 sec). All results are expressed as mean ±SE together with the number of individual experiments (*n*). The statistical significance of differences between mean experimental and control data was assessed by Student's *t*-test.

Results

In the presence of 11.2 mM glucose, oxo-m (0.01-50 µM) provoked a dose-related increase in insulin secretion (Fig 1). Maximal release (0.90 ± 0.09% of islet insulin content per hr; n = 12) was 5 times greater than release in the absence of oxo-m (0.18 ± 0.02% of islet insulin content per hr; n = 12; $P < 0.001$). In the presence of 5.6 mM glucose, 50 µM oxo-m also significantly increased insulin secretion (*not shown*) to values comparable to that found in the presence of 11.2 mM glucose, without oxo-m.

Oxo-m enhances glucose-induced electrical activity in pancreatic β-cells. Figure 2 illustrates the effect of 0.1 and 10 µM oxo-m in the presence of 11.2 mM glucose. Before oxo-m was added to the medium, the cells

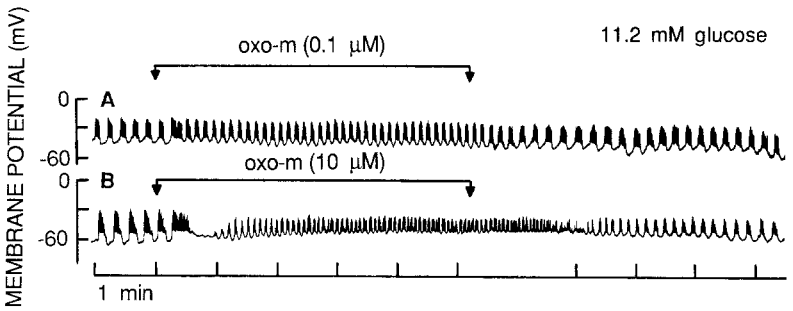

Fig. 2. Effects of oxo-m on glucose-induced electrical activity. Oxo-m, 0.1 μM (*A*) and 10 μM (*B*) was added and removed as indicated by the arrows in the continued presence of 11.2 mM glucose.

showed a typical glucose-induced pattern of electrical activity, periodic slow waves (bursts) of membrane potential (3–5 times/min) on which a fast spike activity was superimposed (Fig. 2, left). Addition of oxo-m to the perfusion medium brought about distinct changes in the burst pattern. In the presence of low concentrations (≤1 μM), oxo-m dose-dependently increased the burst frequency from 4.3 ± 0.3 (n = 14) bursts per min to 4.7 ± 0.8 (n = 4), 5.7 ± 0.5 (n = 5) and 9.4 ± 1.1 (n = 4; $P < 0.01$) bursts per min at 0.01, 0.1 and 1 μM oxo-m, respectively (Fig. 2*A*). The plateau fraction (the relative duration of the bursts) was only slightly increased from 0.46 ± 0.02 (n = 14) to 0.53 ± 0.02 (n = 4) by 1.0 μM oxo-m ($P < 0.05$). In contrast, high concentrations of oxo-m (≥1 μM) induced a multiphasic change in the pattern of electrical activity, including a short period of constant spike activity, which was always followed by a transient inhibition of electrical activity and then a phase of relatively high burst frequency (Fig. 2*B*), reaching 8.5 ± 0.9 (n = 9) bursts per min at 50 μM oxo-m ($P < 0.01$ related to control values) (shown later in Fig. 7). At concentrations of 1 μM or higher, oxo-m induced a small depolarization in the membrane during the silent phase, which was approximately 7 mV more positive than that reached in the absence of oxo-m (in 11 out of 14 cells).

Input resistance measured in the presence of 7 mM glucose during application of 50 μM oxo-m showed a transient decrease followed by a small sustained increase of about 25% over control values in five cells studied.

The effects of 50 μM oxo-m on glucose-induced electrical activity were modified when the medium contained 50 nM Charybdotoxin (ChTX), a specific blocker of the large conductance (maxi) $[Ca^{2+}]_i$–activated K^+ channel, $K_{(Ca)}$, (Fig. 3). The typical initial increase in electrical activity was observed upon addition of the muscarinic agonist, but the transient hyperpolarization was blocked and the silent phase duration decreased to 20 sec (compared to approximately 45 sec in absence of ChTX, Fig. 3*A*). The silent phase was followed by a progressive depolarization and accompanied by high-frequency bursts.

In addition to its effect on electrical activity, ChTX (50 nM) also strongly potentiated insulin secretion; in the presence of 50 μM oxo-m and 11.2 mM glucose, insulin secretion was increased from 0.29 ± 0.05 to 0.43 ± 0.09% of islet insulin content per 30 min ($p < 0.05$).

Figure 4 shows ^{86}Rb efflux from isolated mouse islets. Addition of 50 μM oxo-m to the medium containing 11.2 mM glucose induced a rapid and transient increase in ^{86}Rb efflux from perifused islets. This effect was abolished by ChTX.

To study the influence of muscarinic modulation of cell membrane potential at various glucose levels, 50 μM oxo-m was added in the presence of increasing glucose concentrations (from 0 to 11.2 mM), as shown in Fig. 5. In the absence of glucose, 50 μM oxo-m occasionally induced a slight depolarization of the β-cell, as illustrated in Fig. 5*A*. In the presence of 5.6 mM glucose (Fig. 5*B*), oxo-m caused a transient depolarization of about 15 mV (n = 4) followed by fast oscillations of the membrane potential, but did not induce the usual burst pattern of electrical activity seen at higher glucose concentrations. The average membrane potential at steady state in presence of oxo-m was 8 mV less negative than the control values (n = 4). After raising the glucose concentration to 7 mM (Fig. 5*C*), oxo-m induced several distinct changes in the membrane potential. Initially, the cell depolarized and bursts of spike activity were observed. The electrical activity was then abolished for the remaining period of stimulation, which included an initial hyperpolarization of about 8 mV (n = 3) and a slow return of the membrane potential to near the basal values.

In β-cells perifused with 11.2 mM glucose, 50 μM oxo-m induced a multiphasic change in the electrical activity, as illustrated in Fig. 5*D* (similar to that shown in Fig. 2*B*). After an initial increase in the action potential frequency, a silent period of approximately 40 sec was observed before the burst pattern was restored. Burst frequency was twice that induced by glucose alone, although the plateau fraction remained at about 50% (Fig. 5*D*). Withdrawal of oxo-m at all glucose concentrations was followed by a transient off-response, characterized by depolarization of the cell (up to 10 mV) and/or an increase in the rate of the action potential firing (Fig. 5B-D).

In the presence of 11.2 mM glucose, oxo-m has a similar effect on electrical activity as does the physiological neurotransmitter ACh (Fig. 6). Both agents in-

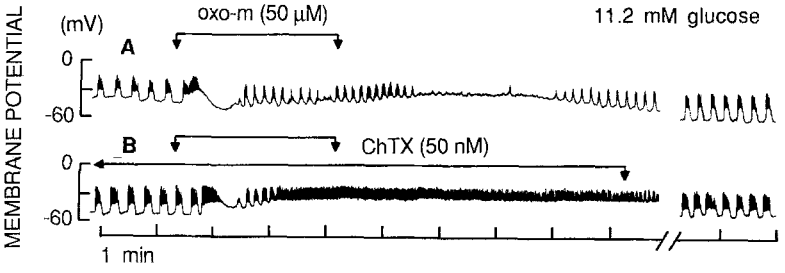

Fig. 3. Inhibition of oxo-m induced polarization by ChTX. (*A*) Oxo-m was added and removed (arrows) in the continued presence of 11.2 mM glucose. (*B*) Oxo-m was added and removed (arrows) in the continued presence of 11.2 mM glucose and 50 nM ChTX. Recovery of control burst pattern was obtained 10 min after removal of oxo-m and ChTX.

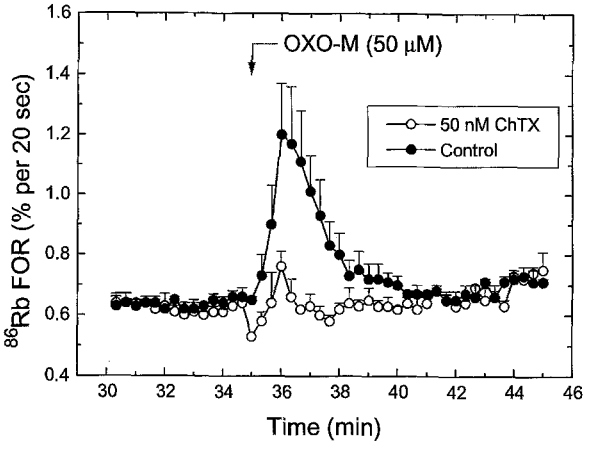

Fig. 4. Effects of ChTX on oxo-m induced increase in ^{86}Rb Fractional Outflow Rate (FOR). Islets were perifused in the continued presence of 11.2 mM glucose. ChTX was added from min 30 to the end of perifusion. Oxo-m was added as indicated. Mean values ± SE of 4 experiments.

duce a triphasic response, first an increase in spike frequency, then a transient hyperpolarization and finally a return to bursting activity at higher frequency; withdrawal of both agonists induces a transient stimulatory off-response. The transient hyperpolarizing action of 50 μM oxo-m (Fig. 6*A*) is greater than that of 100 μM ACh (Fig. 6*B*).

Discussion

The present work presents electrophysiological, ion flux and insulin secretion data to clarify the cellular mechanisms underlying cholinergic stimulation of the pancreatic β-cell.

The results presented here show that oxo-m reproduces the effects of ACh on mouse β-cell electrical activity as expected from a muscarinic agonist. Furthermore, our results show that oxo-m potentiates insulin secretion in a dose-dependent manner over a similar concentration range as in rat islets (Boschero et al., 1995), where it was shown that the muscarinic receptor system in islets of Langerhans is of the M3 subtype (Verspohl et al., 1990, Boschero et al., 1995). Muscarinic stimulation in cells with the M3 receptor subtype induces formation of IP_3, which releases Ca^{2+} from intracellular stores. The consequent increase in $[Ca^{2+}]_i$ as well as the depletion of Ca^{2+} stores may influence β-cell electrical activity and insulin release.

Our results clearly show that muscarinic stimulation has multiple effects on glucose-induced β-cell electrical activity. The ionic mechanisms underlying these effects are discussed below in relation to the actions of muscarinic agonists on intracellular calcium stores.

Initial Hyperpolarization-$[Ca^{2+}]_i$-activated K^+ Channel

At nanomolar concentrations of oxo-m, only an increase in glucose-induced burst frequency occurred; at micromolar concentrations, a transient, marked hyperpolarization (Figs. 2–3 and 5–6) and a transient, marked increase in ^{86}Rb efflux (Fig. 4) were also observed. The input resistance also decreased transiently upon application of micromolar oxo-m when 5.6 mM glucose was present. These data strongly suggest the activation of a potassium conductance (at least transiently) subsequent to addition of oxo-m. Since ChTX inhibits the oxo-m induced hyperpolarization as well as the accompanying increase in ^{86}Rb efflux (Figs. 3 and 4), the large conductance, maxi $K_{(Ca)}$ channel, which is a specific target of ChTX, is likely to be involved in the response. While this channel is rarely active in the presence of glucose alone (Kukuljan, Goncalves & Atwater, 1991) it is possible that increases in cytoplasmic Ca^{2+} concentration owing to muscarinic-stimulated release of stored calcium is sufficient to activate the channel. However, since the oxo-m induced hyperpolarization was not completely suppressed by ChTX, we cannot discard the possibility that inactivation of Ca channels by high cytoplasmic Ca concentrations (Satin et al., 1995) or the simultaneous activation of another type of $K_{(Ca)}$ channel, insensitive to ChTX, are also partially responsible for the transient suppression of action potentials. It was reported that both ChTX sensitive (maxi) (Kukuljan, Goncalves & Atwater, 1991) and ChTX insensitive (Ämmälä, et al., 1991) $K_{(Ca)}$ channels are present in pancreatic β-cells. The ChTX insensitive $K_{(Ca)}$ channel is activated by carbamylcholine (Ämmälä et al., 1991) and hence is a good candidate as a mediator of the ChTX-insensitive portion of the inhi-

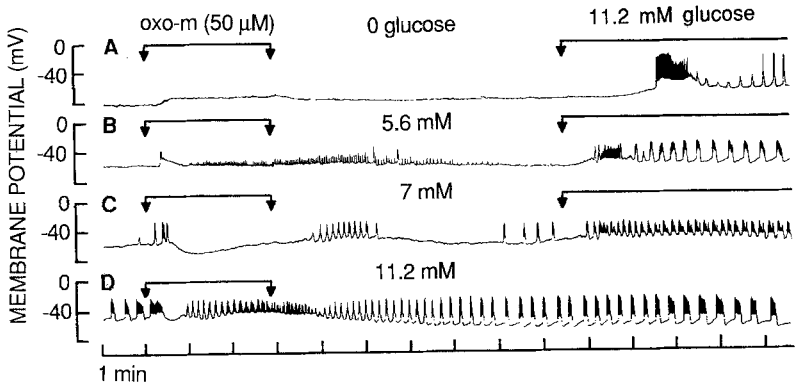

Fig. 5. Effects of oxo-m on electrical activity in the presence of different concentrations of glucose. Oxo-m was added and removed as indicated by the arrows, in the absence of glucose (*A*), or in the presence of 5.6 mM glucose (*B*), 7 mM glucose (*C*) and 11.2 mM glucose (*D*). In *A*, *B*, and *C*, 11.2 mM glucose was added 5 min after removal of oxo-m, as indicated by the arrows at the right side of the Figure. Each record represents a cell from a different islet.

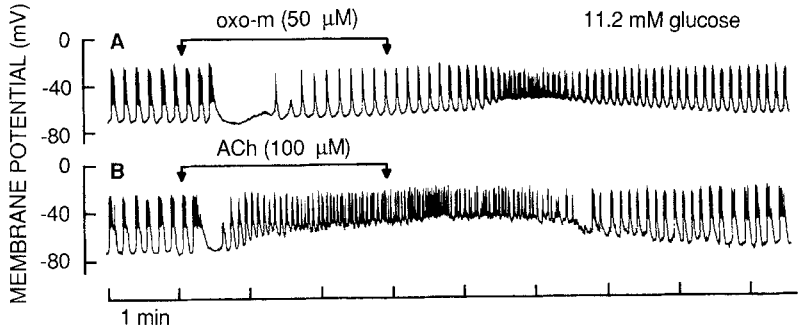

Fig. 6. Comparison of the effects of oxo-m and ACh on electrical activity. (*A*) oxo-m and (*B*) ACh were added and removed, as indicated by the arrows, in the continued presence of 11.2 mM glucose.

bition observed in these experiments. Insulin secretion, measured over 30 min, at 50 μM oxo-m was increased by addition of ChTX, indicating a significant contribution of the maxi $K_{(Ca)}$ channels during muscarinic stimulation of β-cells. Thus, the maxi $K_{(Ca)}$ channel may act as a brake, or negative feedback control, adding some hyperpolarizing current to limit the extent of muscarinic activation. Although ChTX has no effect on steady-state glucose-induced bursting activity (Kukuljan, Goncalves & Atwater, 1991 and Fig. 3), these observations indicate that the maxi $K_{(Ca)}$ channel may be important in the physiology of β-cells in vivo.

Ionic Mechanisms Underlying Steady-state Depolarization

It is clear the the overriding, steady-state effect of oxo-m and other muscarinic agonists (Gagerman, et al., 1978; Santos & Rojas, 1989; Rojas, Santos & Atwater, 1990) is to depolarize the β-cell membrane. This decrease in membrane potential has been ascribed to either a reduction in K^+-permeability (Santos & Rojas, 1989) or an increase in Ca^{2+} and/or Na^+ permeability (Sanches-Andres et al., 1988; Henquin et al., 1988; Hiriart & Ramirez-Medeles, 1993). In the present study, we found that the depolarizing action of oxo-m was associated with a small increase in input resistance, in agreement with the first hypothesis. However, the reduction in input resistance of a cell within the islet may also reflect a decreased gap junctional conductance, not unexpected under conditions of increased $[Ca^{2+}]_i$ (Loewenstein & Rose, 1978; Lazrak & Peracchia, 1993).

The steady-state depolarizing influence of oxo-m may be related to the recent demonstration in several cell types of an ER-factor-activated depolarizing current, CRAC (Calcium Release Activated Current) (Takemura et al., 1989; Putney, 1990; Clementi et al., 1992; Hoth & Penner, 1992; Randriamampita & Tsien, 1993; Parekh, Terlau & Stuhmer, 1993). In these cells, when the muscarinic receptor is activated, IP_3 is produced, and Ca^{2+} is liberated from the ER together with a membrane channel activating factor, CIF (calcium influx factor) (Randriamampita & Tsien, 1993). CIF acts on the membrane to stimulate a depolarizing current which, in nonexcitable cells, is carried by calcium (Putney, 1990). Recent studies indicate that a similar mechanism of communication between calcium-sequestering organelles and the cell membrane may operate in pancreatic β-cells. Thus, thapsigargin (Tg), a blocker of the SERCA (sarco-endoplasmic reticulum calcium ATPase) pump, activates an inward current in single mouse β-cells (Worley III et al., 1994). The current persists in the absence of calcium in the extracellular medium, and Tg also stimulates Mn^{2+} influx in tumoral β-cells (Leech, Holz IV, & Habener, 1994), indicating that unlike that of nonexcitable cells, CRAC in β-cells may be carried by a nonselective cation channel.

In light of these recent findings, the oxo-m stimu-

lated changes in β-cell electrical activity can be interpreted as follows. Release of Ca^{2+} from intracellular stores activates CRAC, resulting in the prolonged depolarized phase at the start of stimulation (*see* for example the first 15 sec after arrow in Fig 5*D* or 6*B*). When $[Ca^{2+}]_i$ increases to high levels (Rojas et al., 1994), owing to continued release of stored Ca^{2+} and/or influx through CRAC channels, $K_{(Ca)}$ is activated (Tabcharani & Misler, 1989). The large hyperpolarizing conductance overrides the relatively small CIF-activated depolarizing current (CRAC) and the membrane potential becomes more negative. The resulting hyperpolarization blocks the action potentials. As $[Ca^{2+}]_i$ is gradually reduced, the balance between CRAC and $K_{(Ca)}$ current then determines the membrane potential. In support of this hypothesis, the experiments with ChTX (illustrated in Figs. 3 and 4) indicate that the maxi $K_{(Ca)}$ (a channel to which no physiological electrical response has been attributed prior to the present study) participates in at least the first 5 min of the effects of muscarinic agonists. Furthermore, Tg, in the presence of glucose, induced a high-frequency burst pattern similar to that observed with oxo-m and ACh (Bertram et al., 1995). Also, CRAC is reported to be voltage-independent in β-cells (Worley III, et al., 1994) and other tissues (Putney, 1990). Finally, the CRAC/$K_{(Ca)}$ scheme is supported by a theoretical study in which the steady-state effects of muscarinic agonists on electrical activity, notably the increase in burst frequency and the depolarization of the silent phase potential, were reproduced when a voltage-independent Ca^{2+} and/or Na^+-carrying channel, CRAC, was added to an existing model of β-cell electrical activity (Bertram et al., 1995; Sherman, 1995). Thus, our data add to the growing evidence that CRAC exists in β-cells, and suggest that the stimulation of electrical activity by oxo-m may reflect enhancement of this voltage-independent, depolarizing, current as a consequence of efflux of Ca^{2+} from the ER.

Muscarinic Activation and Burst Frequency

Many studies have shown the relationship between β-cell electrical activity and insulin secretion (for review, *see* Atwater, Carroll & Li, 1989). Increasing glucose concentration is associated with an increase in the plateau fraction and, hence, in the average frequency of action potentials; this is in turn paralleled by an increase in insulin secretion (Atwater et al., 1989). Variation in burst frequency has been observed as an islet-to-islet variable independent of changes in glucose concentration (Atwater et al., 1980). Muscarinic agonists added at a fixed concentration of glucose offer a unique opportunity to dissociate burst frequency from plateau fraction in the same cell. In particular, oxo-m increases burst frequency without changing plateau fraction (*cf.* Fig. 7*A* and *B*).

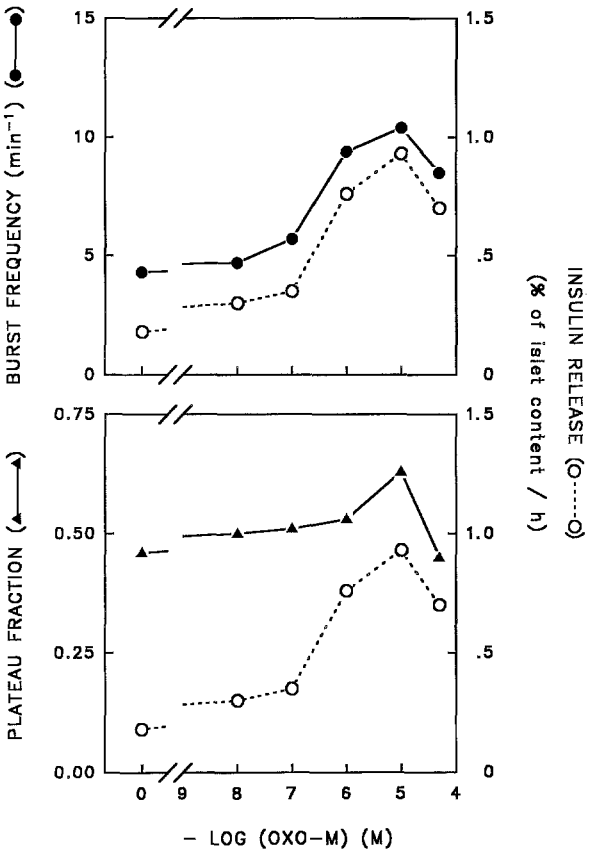

Fig. 7. Relationship between burst frequency (*A*, solid circles) plateau fraction (*B*, solid triangles) with insulin release (*A* & *B*, open circles). Data of insulin release were taken from Fig. 1. and for burst frequency and plateau fraction from the text (*see* Results).

In this study, we show for the first time a close correlation between burst frequency and insulin secretion independent of changes in plateau fraction Fig. 7). The CRAC/$K_{(Ca)}$ model predicts that increases in burst frequency are paralleled by increases in average cytoplasmic $[Ca_i]$, which is consistent with the measurement of higher insulin secretion.

Conclusions

Taken together, our results indicate that there are at least two distinct ionic mechanisms involved in the muscarinic action in the β-cell: (i) an increase in K^+ conductance, some of which seems to be mediated by maxi $K_{(Ca)}$ channels activated in response to the increase in $[Ca^{2+}]_i$ produced by IP_3-dependent intracellular Ca^{2+} release, and (ii) an increase in Na^+ and Ca^{2+} permeability, possibly through a current (CRAC) activated by a factor (CIF) released from ER when Ca^{2+} is released. Furthermore, we propose here that this current, CRAC, may be the ionic mechanism which explains the increase in burst frequency measured in response to muscarinic activation.

Finally, we have shown that insulin secretion is correlated not only with plateau fraction but also with burst frequency.

We thank Dr. Mirta Szpak-Glasman for collaboration during the data analysis and Drs. David Mears, Arthur Sherman, Paul Smolen and Richard Bertram for critical reading of the manuscript. This work was supported in part by Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (CNPq), Coordenadoria de Aperfeicoamento do Ensino Superior (CAPES), and Fundacao de Amparo a Pesquisa do Estado de Sao Paulo (FAPESP), Brazil.

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