Muscarinic modulation of voltage-dependent Ca\textsuperscript{2+} channels in insulin-secreting HIT-T15 cells

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Acetylcholine (ACh) alone has little effect on insulin secretion and β-cell electrical activity yet potentiates insulin secretion in the presence ongoing cyclic electrical activity initiated by glucose or other depolarizing secretagogues. The secretory effects are paralleled by both a depolarization of the plateau potentials and an increased frequency of these Ca\textsuperscript{2+}-dependent potentials as well as the superimposed Ca\textsuperscript{2+} spikes (6, 9, 14). Because glucose-stimulated β-cell electrical activity, insulin secretion, and the effects of ACh on them persist in tetrodotoxin- and Na\textsuperscript{+}-free solutions, a significant part of the ACh effect is likely to be due to an increase in Ca\textsuperscript{2+} channel activity (11–13, 15, 19, 20, 26, 27). Although muscarinic depolarization of β-cells resulting from a decrease in resting K\textsuperscript{+} conductance has been reported (27) and could explain the increased Ca\textsuperscript{2+} influx and insulin secretion, the possibility of a membrane potential-independent activation of Ca\textsuperscript{2+} channels by muscarinic agonists has not been studied in detail. Such a direct activation of β-cell Ca\textsuperscript{2+} currents has been demonstrated for agents that increase intracellular adenosine 3',5'-cyclic monophosphate (cAMP) levels (1).

The mechanism of cholinergic modulation of β-cell electrical activity and its relationship to insulin secretion are poorly understood. However, it is well established that ACh, acting via G protein-coupled muscarinic receptors, stimulates phospholipid hydrolysis, yielding inositol trisphosphate (IP\textsubscript{3}) and diacylglycerol (DAG) (34). The activation of protein kinase C (PKC) by DAG results in the phosphorylation of endogenous protein substrates (8, 10) and appears to be required for the sustained, Ca\textsuperscript{2+}-dependent cholinergic potentiation of glucose-stimulated insulin secretion (16, 22, 23). In contrast, IP\textsubscript{3} production causes only a transient stimulation of insulin secretion, and only at high concentrations of cholinergic agonist (34). Protein kinase A, which is not stimulated by ACh, potentiates glucose-stimulated electrical activity and insulin secretion in a similar manner. These effects have been attributed to both an increased Ca\textsuperscript{2+} channel activity at a given membrane potential and a sensitization of the secretory process to Ca\textsuperscript{2+} (1, 24).

These observations led us to hypothesize that ACh, acting via PKC, could alter the gating of voltage-dependent Ca\textsuperscript{2+} channels activated by membrane depolarization. To test this hypothesis, we used the clonal β-cell line HIT-T15. This continuous cell line provided a homogeneous population of insulin-secreting cells that exhibited all the salient electrophysiological and biochemical features of pancreatic β-cells (15–17, 25). Cell-attached patch recordings of single Ca\textsuperscript{2+} channel Pancreatic β-cells respond to increasing concentrations of extracellular glucose with gradual membrane depolarization until, at 6–8 mM glucose, a threshold is reached for the initiation of cyclic electrical activity. This activity consists of prolonged, voltage-dependent action potentials (plateau potentials) with superimposed spikes, both of which are Ca\textsuperscript{2+}-dependent, interspersed between repolarized, silent phases. Both the duration of the plateau potentials and the fraction of a given cycle spent at this potential are strongly glucose dependent and show a close correlation with Ca\textsuperscript{2+} influx and insulin secretion (20). Subsequent patch-clamp studies have revealed that glucose metabolism results in the inhibition of ATP-sensitive K\textsupersensitive K\textsuperscript{+} channels, leading to membrane depolarization, activation of high-threshold voltage-dependent Ca\textsuperscript{2+} channels, and Ca\textsuperscript{2+} influx essential for stimulus-secretion coupling (2, 3, 36).
currents and permeabilized-patch recordings of whole cell currents were used to study the effects of a muscarinic agonist under conditions that preserved the cytoplasmic integrity of the cells and allowed the control of membrane potential.

**METHODS**

Cell culture. HIT-T15 cells were obtained from the American Type Culture Collection and grown in RPMI-1640 containing 11.1 mM glucose and supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cultures were maintained in a warm (37°C), humidified atmosphere containing 95% air-5% CO2. Cells (passages 59–75) were grown on glass coverslips for patch-clamp recordings. At least 30 min before patch-clamp recordings, coverslips were removed from the culture medium and placed in a glucose-free bath solution containing (in mM) 135 NaCl, 5 KCl, 2 MgCl2, 5 CaCl2, and 5 N-2-hydroxyethylpipеразин-N’-2-этиленафосфонная кислота (HEPES)-NaOH (pH 7.4). This ensured that any residual effects of glucose on Ca2+ channel activity (3, 31) were avoided.

Patch-clamp recordings. Single Ca2+ channel currents were recorded from cell-attached patches in a glucose-free bath solution containing (in mM) 115 KCl, 28 KOH, 1 MgCl2, 1 CaCl2, 10 ethylene glycol-bis(β-aminopropyl ether)-N,N,N’-tetraacetic acid, and 10 HEPES (pH 7.4). This solution closely approximates the cytoplasmic K+ concentration and depolarizes the cells to a membrane potential of ~0 mV. Under these conditions, any depolarization resulting from application of BCh was prevented, and changes in channel activity were ascribed to a membrane potential-independent change in channel kinetics. Gigaohm seals were made using polystyrene-coated glass (Corning 7052) pipettes containing a solution of (in mM) 100 BaCl2, 10 tetraethylammonium chloride (TEA·Cl), and 10 HEPES-Ba(OH)2 (pH 7.4). This solution blocked all K+ currents in the patches while allowing Ba2+ to serve as the carrier of inward current and minimizing the Ca2+-dependent inactivation of channel activity reported in HIT cells (17). Membrane patches were voltage clamped using an Axopatch-1B amplifier (Axon Instruments, Foster City, CA), and the records were stored on computer hard disk. Currents were filtered at 1–3 kHz using an eight-pole Bessel filter and a digitization rate of 5–15 kHz. Capacity and leak subtraction were provided by a dropyridine compound BAY-K-8644 and nifedipine were obtained from Research Biochemicals (Natick, MA).

**RESULTS**

Single Ca2+ channel currents in cell-attached patches. Cell-attached patches exhibited single Ca2+ channel (Ba2+) currents in over 85% of the cells tested. After seal formation, the patches were hyperpolarized to pipette potentials of ~60 to ~100 mV, and channels were activated by 50- to 250-ms depolarizing voltage steps to pipette potentials up to +10 mV. Brief, low-frequency channel openings with a mean single-channel conductance of 26 ± 1 pS (n = 11) were observed only at pipette potentials positive to ~40 mV (Fig. 1). Kinetic analysis of the channel openings revealed a single apparent mean open time (tO) of 0.5 ± 0.1 ms and two apparent mean closed times (tC1, tC2) of 1.3 ± 0.2 and 32.7 ± 6.5 ms, respectively (n = 11). Marked increases in Fo occurred only at pipette potentials positive to ~20 mV (Fig. 2), and the threshold for these channel openings was unaffected by increasing the holding potential to values up to ~100 mV. In addition, these channels exhibited little inactivation during voltage steps lasting several hundred milliseconds to several seconds. Excision of the patches to form inside-out patches resulted in a complete loss of activity within several minutes.

We also tested the dihydropyridine sensitivity of these high-threshold Ca2+ channels. Bath application of the dihydropyridine agonist BAY-K-8644 (0.1–1 µM) caused a dramatic change in the Ca2+ channel gating pattern to one of frequent, prolonged openings (Fig. 3). This resulted in a significant increase in tavg from 0.04 ± 0.01 to 0.56 ± 0.19 nA (P ≤ 0.05; n = 5). In contrast, the dihydropyridine antagonist nifedipine (1–5 µM) significantly reduced tavg from 0.05 ± 0.01 to 0.01 ± 0.01 pA (P ≤ 0.05; n = 6), and the remaining channel currents exhibited the same thresholds and single-channel conductances recorded in the absence of the antagonist (Fig. 4).
MUSCARINIC ACTIVATION OF Ca\textsuperscript{2+} CHANNELS

Macroscopic Ca\textsuperscript{2+} channel currents in permeabilized patches. Whole cell recordings in the permeabilized-patch configuration were used to confirm that the properties of the single Ca\textsuperscript{2+} channel currents accurately reflected the properties of macroscopic Ca\textsuperscript{2+} channel currents. Like the single-channel currents, whole cell currents exhibited a threshold positive to −40 mV, with most channel activity occurring at pipette potentials positive to 0 mV. The threshold was not changed by increasing the holding potential from −70 to −100 mV. The mean peak current of 105 ± 13 pA (n = 6) occurred at +10 mV and reversed at +50 mV. Application of nifedipine (5 µM) significantly decreased mean peak current by 84% (from 91 ± 12 to 15 ± 4 pA) in all three cells tested.

Effects of BCh on Ca\textsuperscript{2+} channel currents. Single Ca\textsuperscript{2+} channel currents were activated by depolarizing voltage steps (40–80 mV; 0.1–0.5 Hz) from a holding potential of −70 mV. Under control conditions, single Ca\textsuperscript{2+} channel openings of HIT-T15 cells occurred as low-frequency bursts described by τ\textsubscript{o}, τ\textsubscript{c1}, and τ\textsubscript{c2}. Exposure of HIT-T15 cells to the muscarinic agonist BCh (100 µM) caused a large increase in the frequency of channel openings (Fig. 5) in 27 of 31 patches tested. Statistically significant (P ≤ 0.05) increases in τ\textsubscript{o}, F\textsubscript{o}, and I\textsubscript{avg} as well as significant decreases in the longer of two apparent mean closed times, τ\textsubscript{c2}, accompanied the increased activity (Table 1). These effects occurred within 5 min of changing the bath solution and persisted throughout the duration of exposure (tested up to 45 min after completion of bath change). In contrast, all cells exposed to BCh after pretreatment with atropine (10 µM; n = 6) exhibited no significant changes in F\textsubscript{o} (0.01 vs. 0.01) or I\textsubscript{avg} (0.01 vs. 0.01 pA/ms), whereas, in the same passage of cells, two of three cells responded to BCh in the absence of atropine with significant increases in F\textsubscript{o} (0.02 vs. 0.06) and I\textsubscript{avg} (0.02 vs. 0.06 pA/ms). Pretreatment with PTX (100 ng/ml) for 12–48 h before recording had no effect on the BCh response. In four of five cells tested, BCh (100 µM) caused significant increases in F\textsubscript{o} (0.03 vs. 0.07) and I\textsubscript{avg} (0.04 vs. 0.08 pA/ms). In recordings from cells of the same passage not treated with PTX, BCh significantly increased F\textsubscript{o} (0.03 vs. 0.08) and I\textsubscript{avg} (0.04 vs. 0.08 pA/ms) in three of four cells tested.

Similar effects of BCh on whole cell Ca\textsuperscript{2+} channel currents were measured using permeabilized-patch recordings (Fig. 6). In all five cells tested, BCh (100 µM) significantly increased the mean peak current from 100 ± 14 to 197 ± 27 pA. As shown in Fig. 6, the effect of BCh was observed only at test potentials positive to −30 mV, with the greatest increases in current occurring at test potentials positive to −20 mV.

Activation of PKC mimics BCh effects. Because PKC has been implicated in muscarinic potentiation of glucose-stimulated insulin secretion (16, 22, 23), we compared the effects of exogenous activators of the enzyme with those of BCh. Acute application of the active

![Fig. 1. Activation of single Ca\textsuperscript{2+} channel currents in a cell-attached patch from an HIT-T15 cell. Single-channel currents were recorded in a high (143 mM)-K\textsuperscript{+} bath solution, using pipettes filled with a solution that contained 100 mM BaCl\textsubscript{2} and 10 mM TEA·Cl. Pipette potential was stepped from a holding potential of −90 mV to potentials indicated at right. In all patches tested, threshold for channel activation was positive to −40 mV, with most channel activation occurring positive to −20 mV. Channel openings were observed throughout duration of voltage steps, and simultaneous opening of at least 2 channels occurred at a step potential of 0 mV.](http://ajpgi.physiology.org/)

![Fig. 2. HIT-T15 cells exhibited only high-threshold, voltage-dependent Ca\textsuperscript{2+} channels. In cell-attached patches, channel openings were recorded during a series of 10-mV depolarizing voltage steps from a holding potential of −100 mV, using solutions described in Fig. 1 legend. Threshold for channel activation occurred positive to −40 mV, with most of channel activity occurring at pipette potentials positive to −20 mV. Fractional open time (F\textsubscript{o}) was fraction of total trial time (20–67 ms sweeps at each test potential) during which at least 1 channel was open. Each point represents mean ± SE of F\textsubscript{o} recorded from 6 patches.](http://ajpgi.physiology.org/)
phorbol ester PMA (10–100 nM) resulted in a marked increase in the frequency of Ca$^{2+}$ channel openings (Fig. 7). Similar to the response to BCh, the increased channel activity was accompanied by significant ($P < 0.05$) increases in $F_0$ ($0.03 \pm 0.01$ vs. $0.14 \pm 0.02$) and $I_{avg}$ ($0.03 \pm 0.01$ vs. $0.14 \pm 0.03$ pA/ms) and a significant decrease in $\tau_2$ ($27.1 \pm 3.8$ vs. $12.8 \pm 1.6$ ms), but $\tau_0$ ($0.4 \pm 0.1$ vs. $0.5 \pm 0.1$ ms) and $\tau_{cl}$ ($2.7 \pm 0.7$ vs. $1.7 \pm 0.2$ ms) were unchanged in six of eight patches tested.

Depletion or inhibition of PKC activity antagonizes effects of BCh. To further test the role of PKC in muscarinic activation, we examined the effects of depleting or inhibiting PKC activity. Depletion of PKC activity was accomplished by chronic (20–32 h) exposure to PMA (200 nM), a treatment known to deplete enzyme activity by $>75\%$ in β-cells (25). After such treatment, BCh caused only a small (20%) but significant increase in $F_0$ and $I_{avg}$ and no change in the mean open or closed times in 9 of 12 cells tested. In contrast, seven of nine cells chronically exposed to 4α-phorbol (200 nM) responded to BCh with large increases in $F_0$ (180%) and $I_{avg}$ (225%) and a decrease (54%) in $\tau_2$ (Table 2). The nature and magnitude of these effects were comparable to those observed previously in untreated cells (Table 1).

G400 MUSCARINIC ACTIVATION OF Ca$^{2+}$ CHANNELS
CONTROL

- - - -

BCH

- - - -

2 pA 10 msec

Fig. 5. Muscarinic agonist bethanechol (BCh) increased high-threshold, single Ca\(^{2+}\) channel currents independent of a change in membrane potential. Top: single-channel currents were recorded from a cell-attached patch in a high-K\(^+\) bath solution that chemically clamped membrane potential at or close to 0 mV. Channel openings were elicited by depolarizing voltage steps from a holding potential of −70 mV to a test potential of −15 mV. Under these conditions, channel openings were brief and infrequent. Bottom: 10-min exposure to a bath solution that contained 100 µM BCh markedly increased Ca\(^{2+}\) channel activity, and \(I_{\text{avg}}\), calculated from 100 sweeps, increased from 0.03 to 0.06 pA/ms.

We also examined the effects of BCh in the presence of the protein kinase inhibitor staurosporine (100 nM). This concentration of staurosporine has been reported to inhibit islet PKC activity by >80% (12). Ca\(^{2+}\) channel activity was initially recorded in the presence of staurosporine alone before switching to a solution containing both staurosporine and BCh (100 µM). Under these conditions, BCh evoked small but significant increases in \(F_0\) (0.03 ± 0.01 vs. 0.04 ± 0.01) and \(I_{\text{avg}}\) (0.04 ± 0.01 vs. 0.05 ± 0.01 pA/ms), but no changes in \(\tau_{o}\) (0.3 ± 0.1 vs. 0.3 ± 0.1 ms), \(\tau_{c1}\) (2.1 ± 0.3 vs. 1.9 ± 0.5 ms), or \(\tau_{c2}\) (24.6 ± 4.2 vs. 23.3 ± 5.9 ms) were observed in seven of nine cells tested. In parallel control cells from the same passage, BCh, in the presence of the vehicle (0.01% DMSO) alone, caused significant increases in \(F_0\) (0.04 ± 0.01 vs. 0.10 ± 0.02) and \(I_{\text{avg}}\) (0.05 ± 0.01 vs. 0.10 ± 0.02 pA/ms) accompanied by a significant decrease in \(\tau_{c2}\) (15.9 ± 1.6 vs. 8.4 ± 2.2 ms), but \(\tau_{o}\) (0.3 ± 0.1 vs. 0.4 ± 0.1 ms) and \(\tau_{c1}\) (1.9 ± 0.6 vs. 1.2 ± 0.2 ms) were unchanged.

DISCUSSION

Ca\(^{2+}\) channels in HIT-T15 cells. HIT-T15 cells exhibited exclusively high-threshold voltage-dependent Ca\(^{2+}\) channel currents. Both single-channel and whole cell currents had thresholds of activation positive to −40 mV that were unaltered when the holding potential was increased from −60 to −100 mV. These high-threshold currents were predominantly, if not exclusively, L-type currents. Consistent with this conclusion were blockade of >80% of single-channel and whole cell currents by nifedipine, a mean single-channel conductance of 26 pS, little current inactivation during prolonged depolarizations, and rapid, complete loss of single-channel currents after excision of cell-attached patches. Using the solutions and recording protocols that revealed low-threshold, T-type channels in rat β-cells (3), we observed no such channels in cell-attached patches from HIT cells. Our results are the first to characterize single Ca\(^{2+}\) channel currents in HIT-T15 cells and to correlate them with whole cell currents recorded under conditions that preserved the normal cytosolic components required for Ca\(^{2+}\) channel activity.

Using standard whole cell recordings of HIT cell Ca\(^{2+}\) currents, Keahey et al. (17) reported thresholds between −50 and −40 mV from a holding potential of −80 mV. These currents were reduced by ~85% in the presence of nimodipine, and the residual currents retained the kinetics of L-type Ca\(^{2+}\) currents. Parallel studies of the dihydropyridine sensitivity of K\(^+\)-stimulated insulin secretion and intracellular Ca\(^{2+}\) accumulation revealed a strong correlation with that of the Ca\(^{2+}\) currents. These authors concluded that HIT cells contain almost exclusively high-threshold, L-type Ca\(^{2+}\) channels and that, as reported in islets and primary cultures of β-cells, the entry of extracellular Ca\(^{2+}\) essential for insulin secretion occurs through these channels. Simultaneous measurements of Ca\(^{2+}\) transients and whole cell Ca\(^{2+}\) currents in HIT cells also indicated that voltage-dependent influx was carried exclusively by L-type channels (7). Our results are the first to confirm these conclusions at the single-channel level.

Fast, Ca\(^{2+}\)-dependent and slow, voltage-dependent components of Ca\(^{2+}\) current inactivation have been...

Table 1. Effects of bethanechol on single Ca\(^{2+}\) channel kinetics in HIT-T15 cells

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>BCh</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\tau_{o}), ms</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1*</td>
</tr>
<tr>
<td>(\tau_{c1}), ms</td>
<td>2.1 ± 0.3</td>
<td>1.7 ± 0.1</td>
</tr>
<tr>
<td>(\tau_{c2}), ms</td>
<td>25.9 ± 2.8</td>
<td>145.1 ± 16.8*</td>
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<tr>
<td>(F_0)</td>
<td>0.03 ± 0.01</td>
<td>0.09 ± 0.01*</td>
</tr>
<tr>
<td>(I_{\text{avg}}), pA/ms</td>
<td>0.04 ± 0.01</td>
<td>0.09 ± 0.01*</td>
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Values are means ± SE; \(n = 27\). Bethanechol (BCh, 100 µM), applied for 10–30 min, increased single Ca\(^{2+}\) channel activity in 27 of 31 cells tested. \(\tau_{o}\), mean open time; \(\tau_{c1}\), \(\tau_{c2}\), mean closed times; \(F_0\), fractional open time; \(I_{\text{avg}}\), mean current. *Significantly different (\(P \leq 0.05\)) using 2-tailed Student’s paired t-test.
described in HIT cells (28). Currents elicited at more negative test potentials (−50 to −30 mV) were either unaffected or increased by cell dialysis, whereas currents observed at more positive potentials were subject to rundown, suggesting the presence of high-threshold L- and N-type channels. An ω-conotoxin (CTX)-sensitive, high-threshold (N-type) Ca$^{2+}$ current has also been recorded in the rat insulinoma cell line RINm5F (30). This high-threshold current comprised 15–25% of the total Ca$^{2+}$ current, and its blockade with CTX inhibited stimulated insulin secretion by up to 51%. Although our experiments cannot prove that such a current does not exist in HIT cells, our results indicate that it could contribute only a small fraction (<20%) of the total current, whereas in HIT cells over 90% of insulin secretion is dihydropyridine sensitive (17). Thus the effects of BCh and PKC activators on Ca$^{2+}$ currents in HIT cells can be ascribed to actions on high-threshold, L-type channels.

Muscarinic agonist increased voltage-activated Ca$^{2+}$ channel currents in HIT-T15 cells. Stimulation of muscarinic receptors increased single-channel and whole cell L-type Ca$^{2+}$ channel currents two- to threefold in HIT-T15 cells under recording conditions that chemically or electrically clamped resting membrane potential. This is the first direct evidence for a muscarinic receptor-mediated modulation of single Ca$^{2+}$ channel currents in pancreatic β-cells that is independent of effects on resting membrane potential. Antagonism of the BCh effects by atropine confirmed that this was a muscarinic receptor-mediated event, and the PTX-resistant nature of the effects suggests coupling of the receptor to G proteins other than G$i$ or G$o$, which are associated with PTX-sensitive inhibition of L-type Ca$^{2+}$ channels in β-cells (29). These observations are consistent with previous reports that cholinergic potentiation of glucose-stimulated insulin secretion is mediated by PTX-resistant G proteins (30, 34). Recent work indicates that muscarinic M$3$ receptors mediate cholinergic potentiation of glucose-stimulated insulin secretion (5), and in other tissues, these receptors appear preferentially coupled to G$q$ and G$11$ and lead to the activation of phospholipase C$\beta$ (18). The specific stimulatory G proteins mediating cholinergic activation of phospholipase C in islets remain to be determined; however, one recent study has implicated G$o$ (33).

Muscarinic stimulation of Ca$^{2+}$ currents required PKC activation. Cholinergic agonists activate phospholipase C with the production of IP$_3$ and DAG in both pancreatic islet cells and HIT cells. These two second messengers respectively cause the release of intracellular Ca$^{2+}$ and the activation of PKC (8, 15, 16, 20, 22, 23, 34, 36). In HIT-T15 cells activation of PKC, using either a phorbol ester or a DAG analog, qualitatively and
quantitatively mimicked the effects of BCh on single and whole cell Ca\textsuperscript{2+} channel currents. Conversely, both the PKC inhibitor staurosporine and depletion of PKC activity by chronic phorbol treatment markedly inhibited the effects of BCh. Biochemical studies of islet and HIT cells have demonstrated that staurosporine, at the concentrations used in our experiments, inhibited PKC activity by 70–80\% (37), whereas chronic exposure to phorbol esters reduced PKC activity by 75\%. These effects were accompanied by a comparable inhibition of the sustained cholinergic potentiation of glucose-stimulated insulin secretion that was dependent on Ca\textsuperscript{2+} influx (16, 22, 23, 32). We observed a comparable inhibition of BCh-stimulated increases in Ca\textsuperscript{2+} currents. Thus intact PKC activity appears essential for the muscarinic effects that we observed.

These results do not preclude the possibility that PKC might activate another second-messenger system, which in turn modulates Ca\textsuperscript{2+} channels. cAMP has been shown to increase Ca\textsuperscript{2+} influx through voltage-dependent channels in \(\beta\)-cells (1, 24), and we have also recorded significant (\(P \leq 0.05\)) increases in \(F_o\) (0.04 ± 0.01 vs. 0.10 ± 0.01; 5 of 6 cells) of single Ca\textsuperscript{2+} channel currents in HIT-T15 cells exposed to forskolin (5–10 \(\mu\)M) in the presence of 3-isobutyl-1-methylxanthine (100 \(\mu\)M). The membrane-permeable analog 8-bromo-

Table 2. Effects of chronic exposure to phorbol esters on bethanechol activation of single Ca\textsuperscript{2+} channel currents

\begin{tabular}{|c|c|c|c|c|}
\hline
 & \textbf{4\(\alpha\)-Phorbol} & & & \\
 & Control & BCh & Control & BCh \\
\hline \hline
\(\tau_\text{p}, \text{ ms}\) & 0.5 ± 0.1 & 0.6 ± 0.1 & 0.5 ± 0.1 & 0.5 ± 0.1 \\
\(\tau_\text{d}, \text{ ms}\) & 1.9 ± 0.4 & 1.4 ± 0.2 & 1.8 ± 0.3 & 2.1 ± 0.3 \\
\(\tau_\text{d}, \text{ ms}\) & 24.1 ± 3.7 & 11.0 ± 1.7* & 26.3 ± 5.2 & 23.3 ± 4.8 \\
\(F_o\) & 0.05 ± 0.01 & 0.14 ± 0.03* & 0.05 ± 0.01 & 0.06 ± 0.01 \\
\(F_{\text{avg}}, \text{ pA/ms}\) & 0.04 ± 0.01 & 0.13 ± 0.04* & 0.05 ± 0.01 & 0.06 ± 0.01 \\
\hline
\end{tabular}

Values are means ± SE. HIT-T15 cells from same passage were grown in culture medium supplemented with either 4\(\alpha\)-phorbol or phorbol 12-myristate 13-acetate (PMA, 200 nM) for 20–32 h before effects of BCh (100 \(\mu\)M) were tested. BCh significantly increased channel activity in 7 of 9 cells grown in 4\(\alpha\)-phorbol, whereas 9 of 12 cells grown in PMA responded with a small but insignificant increase in activity. *Significantly different (\(P \leq 0.05\)) using 2-tailed Student's paired t-test.
cAMP (2 mM) also increased F₀ (0.05 ± 0.01 vs. 0.09 ± 0.03; 3 of 3 cells) in HIT-T15 cells (unpublished observations). However, numerous biochemical studies on islet cells and clonal β-cell lines have demonstrated muscarinic potentiation of glucose-stimulated insulin secretion without any effect on cAMP levels (9, 20, 36). The fact that our experiments were conducted with the cells depolarized to a membrane potential at or near 0 mV in the presence of extracellular Ca²⁺ argues against the activation of another protein kinase by BCh due to increased Ca²⁺ influx. Our experiments also cannot rule out some direct modulation of Ca²⁺ channel activity by interaction with G protein subunits.

Potential importance in insulin secretion. Numerous studies have confirmed that cholinergic agonists produce a biphasic potentiation of glucose-stimulated insulin secretion. An initial rapid, brief increase in secretion, mediated by IP₃ generation and release of intracellular Ca²⁺, is followed by a larger, prolonged PKC-mediated increase in secretion that is dependent on extracellular Ca²⁺ (11, 15, 16, 20, 22, 23, 25, 32, 34, 36). Previous work indicated that cholinergic agonists, in the presence of substimulatory concentrations of glucose, depolarized the membrane potential of β-cells (6, 9, 20, 27) and increased Na⁺ influx (12) but potentiated cyclic electrical activity, Ca²⁺ influx, or insulin secretion only in the presence of ongoing electrical activity initiated by glucose or some other depolarizing secretagogue (9, 11, 14, 16, 20, 26, 36). To date, two mechanisms have been postulated for the PKC-mediated effects: an increase in Ca²⁺ influx secondary to membrane depolarization, and a sensitization of the secretory process to Ca²⁺ (6, 9, 11, 16, 22, 23, 26, 27). Our results suggest a third, PKC-mediated, cholinergic mechanism, an increase in Ca²⁺ channel activity at any given membrane potential above the threshold for channel opening. Given the dose correlation of intracellular Ca²⁺ concentration and insulin secretion (20, 36), an increase in the Ca²⁺ currents would be expected to contribute to increased insulin secretion. It is likely that all three mechanisms, membrane depolarization, direct Ca²⁺ channel activation, and sensitization of exocytosis, act in concert during cholinergic potentiation of glucose-stimulated insulin secretion.

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