Voltage-dependent delayed rectifier K\(^+\) channels regulate aspects of both stimulus-secretion and excitation-contraction coupling, but assigning specific roles to these channels has proved problematic. Using transgenically derived insulinoma cells (βTC3-neo) and β-cells purified from rodent pancreatic islets of Langerhans, we studied the expression and role of delayed rectifiers in glucose-stimulated insulin secretion. Using reverse-transcription polymerase chain reaction methods to amplify all known candidate delayed rectifier transcripts, the expression of the K\(^+\) channel gene Kv2.1 in βTC3-neo insulinoma cells and purified rodent pancreatic β-cells was detected and confirmed by immunoblotting in the insulinoma cells. βTC3-neo cells were also found to express a related K\(^+\) channel, Kv3.2. Whole-cell patch clamp demonstrated the presence of delayed rectifier K\(^+\) currents inhibited by tetrathylationmonium (TEA) and 4-aminopyridine, with similar \(K_v\) values to that of Kv2.1, correlating delayed rectifier gene expression with the K\(^+\) currents. The effect of these blockers on intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) was studied with fura-2 microspectrofluorimetry and imaging techniques. In the absence of glucose, exposure to TEA (1–20 mM) had minimal effects on βTC3-neo or rodent islet [Ca\(^{2+}\)]\(_i\), but in the presence of glucose, TEA activated large amplitude [Ca\(^{2+}\)]\(_i\) oscillations. In the insulinoma cells the TEA-induced [Ca\(^{2+}\)]\(_i\) oscillations were driven by synchronous oscillations in membrane potential, resulting in a 4-fold potentiation of insulin secretion. Activation of specific delayed rectifier K\(^+\) channels can therefore suppress stimulus-secretion coupling by damping oscillations in membrane potential and [Ca\(^{2+}\)]\(_i\) and thereby regulate secretion. These studies implicate previously uncharacterized β-cell delayed rectifier K\(^+\) channels in the regulation of membrane repolarization, [Ca\(^{2+}\)]\(_i\), and insulin secretion.
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produce membrane repolarization, lowering of [Ca\(^{2+}\)]\(_i\) and thereby deplete insulin secretion.

**EXPERIMENTAL PROCEDURES**

**Growth of Insulinoma Cells—\(\beta\)TC3 cells were cultured essentially as originally described (21). A clonal subline (\(\beta\)TC3-neo) was isolated after transfection with \(\beta\)TC3-neo by electroporation (22), and this \(\beta\)TC3-neo line was maintained in the continued presence of 1 mg/ml G418 (Life Technologies, Inc.). Cells were seeded for 4–6 days in Dulbecco’s modified Eagle’s medium supplemented with 15% normal horse serum, 2.5% fetal calf serum, 50 units/ml penicillin, and 50 \(\mu\)g/ml streptomycin. Eighteen hours prior to experiments, medium was changed to RPMI 1640 containing 0.1 mM CaCl\(_2\). Cells were dissociated in 2.5% trypsin with gentle trituration. The recovered single cell pellet was resuspended in EH containing 1 mM EGTA and introduced into the sample stream of a flow medium (EH, Ref. 24) with deoxyribonuclease and trypsin with gentle stirring (22, 25).

**Fluorescence-activated Cell Sorting of Rodent \(\beta\) Cells—Fluorescence-activated cell sorting (FACS) was employed to isolate \(\beta\) cells essentially as described (24). Islets were dissociated in Ca\(^{2+}\)-free Earle’s HEPES medium (EH, Ref. 24) with deoxyribonuclelease and trypsin with gentle trituration. The recovered single cell pellet was resuspended in EH containing 1 mM EGTA and introduced into the sample stream of a flow cytometer (Becton Dickinson FACStarPlus) equipped with an argon ion laser in the primary light path (90 mW at 488 nm, Ion Laser Technology, Salt Lake City, UT). Events were analyzed and collected on a forward scatter trigger. Signals and side scatter were displayed and collected via a 530/30 nm band-pass filter following transit through a 560 nm short-pass dichroic filter. The integrity of the cell preparation was checked by propidium iodide exclusion. The propidium iodide fluorescence signal was collected by transit of a 660/20 nm band-pass filter receiving signal from a 610 nm short-pass dichroic filter. Rectangular sort regions were defined by producing Cartesian plots displaying forward light scatter versus autofluorescence to delineate the \(\beta\) cell population. The processing of the fluorescence signals in the linear mode aided in the resolution of two major cell populations; \(\beta\) cells could be distinguished as a set of cells with elevated green fluorescence, elevated forward and side scatter relative to other cells in the preparation. The \(\beta\) cell populations were sorted in two-drop packets into sterile glass collection tubes containing 2 ml of fetal bovine serum. All cell events with values outside of the sort region were diverted to a separate collection tube. The cytometer target fluorescence values were standardized using glutaraldehyde-fixed chicken red blood cells immediately before and after \(\beta\)TC3 cell sample batches were run. In addition, dual wavelength digitized video fluorescent microscopy with fura-2 in single \(\beta\)TC3-neo cells, pre-loaded with fura-2, were current-clamped using perforated patch techniques as described (5). In some experiments (Table II), dual wavelength digitized video fluorescent microscopy with fura-2 in single \(\beta\)TC3-neo cells was performed using an intensified charge-coupled device (Hamamatsu C2400) and Metafluor imaging software (Universal Imaging).

**Reverse Transcriptase-Polymerase Chain Reaction Amplification (RT-PCR)—\(\beta\)TC3-neo and mouse brain poly(A)\(^+\) RNA was prepared using an oligo-\(\text{dT}\) binding method (Quickprep micro mRNA kit, Pharmacia Biotech Inc.), and first strand was cDNA reverse-transcribed using random primers (Superscript Plus Transcriptase, Life Technologies, Inc.). The polymerase chain reaction (PCR) was then employed (typical protocol: 94°C, 3 min; then 94°C, 1 min, 52°C, 30 s, 72°C, 30 s for 30 cycles) for each of the primer pairs shown in Table I. In some experiments, the annealing temperature was reduced to 48°C. Amplification experiments were performed using two positive controls, rodent brain cDNA with channel primers and insulinoma cell cDNA with proinsulin primers. DNA sequencing confirmed the closest matches of the amplified sequences were to \(\beta\)TC3-neo cells, preloaded with fura-2, were current-clamped using perforated patch techniques as described (5). In some experiments (Table II), dual wavelength digitized video fluorescent microscopy with fura-2 in single \(\beta\)TC3-neo cells was performed using an intensified charge-coupled device (Hamamatsu C2400) and Metafluor imaging software (Universal Imaging).

**RESULTS AND DISCUSSION**

While delayed rectifier K\(^+\) channels are known to regulate the membrane potential of electrically excitable cells (26), detailed knowledge of K\(^+\) channel isoform expression and function in insulin-secreting \(\beta\) cells is incomplete. A previous study, performed prior to the identification of multiple families of voltage-activated K\(^+\) channel genes, found transcripts encoding Shaker (Kv1.X)-related K\(^+\) channels in RIN and HIT insulinoma cells and whole islets isolated from leptin-resistant hyperglycemic ob/ob mice (20). In this investigation we used clonal insulinoma cells (\(\beta\)TC3-neo, derived from transgenic animals expressing the SV-40 T-antigen driven by the insulin promoter) and purified rodent \(\beta\) cell preparations to analyze the expression of delayed rectifier family genes with RT-PCR. Kv1 family expression was sought in \(\beta\)TC3-neo cells utilizing oligonucleotide primers (see Table I) designed to amplify the highly conserved pore region (pair A) or the highly conserved domain upstream from the first membrane spanning domain (pair A) followed by individual primer pairs for each member of the Kv1.1-Kv1.7 family. Identification of other families of delayed rectifier K\(^+\) channel genes in \(\beta\)TC3-neo cells relied on the use of isoform-specific oligonucleotide primers to amplify either the pore region or a section of the 3’ end of the coding region. DNA amplification experiments were performed using two positive controls, rodent brain cDNA with channel primers and insulinoma cell cDNA with proinsulin primers. We were unable to find evidence of expression of any of the seven Kv1 isoforms in \(\beta\)TC3 or \(\beta\)TC3-neo cells despite multiple attempts with degenerate and specific primers. Specific amplicons were, however, obtained with primers for Kv2.1 and Kv3.2 (Fig. 1A). DNA sequencing confirmed the closest matches of the amplified sequences were to Kv2.1 and Kv3.2, respectively (Fig. 2), although the sequence of the mouse isoform of Kv3.2 has not yet been published. An antibody specifically recognizing Kv2.1 was employed to show that a membrane preparation from \(\beta\)TC3-neo cells contained an immunoreactive band of 108 kDa as reported previously for Kv2.1 (Fig. 1B) (25).

To extend these results to primary cultured cells, we next determined which Kv family channels were expressed in FACS-purified rodent \(\beta\) cells utilizing the RT-PCR strategy as described above for the \(\beta\)TC3-neo cells (Table I, Fig. 3). The only delayed rectifier cDNA that could be reproducibly amplified from purified rat and mouse \(\beta\) cells was Kv2.1. In some experiments DNA bands indicating Kv1.6, Kv1.2, and Kv2.2 expression were also faintly detected, but these could not be isolated and confirmed by DNA sequencing (data not shown). While a previous study reported expression of Kv1.X genes in...
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All sequences are written 5’ to 3’; “F” indicates “forward” primer and “R” indicates “reverse” primer, and the number at the end indicates the 3’ bp for the sequence indicated.

| Kv family | PairA | PairB |
|-----------|-------|-------|
| Kv1       | (F) ATTGGATCCATC(T)TC(T)TA(A/T)TA(C/T)CA(G/A)TC1GG (498; hKv1.5) | (R) ATTGAATTCA(A/G)TGTC(T)TG(T)CA(G/T)GA1CC (708). |
|           | (F) ATTGAATTCG(A/G)TACTTG(G/A)AA1A(T/G)TACGCC (708). |       |
| Kv2       | (F) GGGAGGTCCAACACATTACA (2272 mKv2.1); | (R) CTCTGAGGTGAGCACT (2598); |
|           | (R) CTCTGAGGAGCACT (2598); |                   |
|           | (R) TAGACGAGAATCGGGAAGAGG (1564); |                   |
|           | (R) GAAAGTTGGCCCGACACACCC (944); |                   |
|           | (R) GTCGTCCTTGTAGAACCGGAGAAGT (1268). |       |
|           | (R) CATCATCACTGCTGCTGGCCCTAC (C/T)CT (968); |                   |
|           | (R) AGAACCGCATGGGGAGATTT (1285); |                   |
| Kv3       | (F) GAAAGTTGGCCCGACACACCCAC (1321, Kv3.2b); | (R) TTCCGGAAAGTGATAATG (1669); |
|           | (R) TTCCGGAAAGTGATAATG (1669); |                   |
|           | (R) AGAACCGCATGGGGAGATTT (1285); |                   |
|           | (R) CATCATCACTGCTGCTGGCCCTAC (C/T)CT (968); |                   |
|           | (R) AGAACCGCATGGGGAGATTT (1285); |                   |
|           | (R) AGAACCGCATGGGGAGATTT (1285); |                   |
|           | (R) AGAACCGCATGGGGAGATTT (1285); |                   |
| Kv4       | (F) GGGAGGTCCAACACATTACA (1189, Kv4.2); | (R) GTGGGGAGAAGTGGGGAAGG (1564); |
|           | (R) GTGGGGAGAAGTGGGGAAGG (1564); |                   |
|           | (R) AGAACCGCATGGGGAGATTT (1285); |                   |
|           | (R) TCCGGAAAGTGATAATG (1669); |                   |
|           | (R) GTGGGGAGAAGTGGGGAAGG (1564); |                   |
|           | (R) AGAACCGCATGGGGAGATTT (1285); |                   |
|           | (R) AGAACCGCATGGGGAGATTT (1285); |                   |
|           | (R) TCCGGAAAGTGATAATG (1669); |                   |
|           | (R) TCCGGAAAGTGATAATG (1669); |                   |
|           | (R) TCCGGAAAGTGATAATG (1669); |                   |
|           | (R) TCCGGAAAGTGATAATG (1669); |                   |
|           | (R) TCCGGAAAGTGATAATG (1669); |                   |

Fig. 1. Analysis of K⁺ channel gene expression in βTC3 cells. A, amplification of βTC3-neo cell cDNA with primers for Kv channels. Lane 1 shows results obtained using a primer pair for Kv2X. Lane 2, primers for Kv3.X. B, immunoblot of membrane proteins detected with Anti-Kv2.1 antibody. Lane 1, mouse brain membranes (5 μg); lane 2, AT20 cell membranes (20 μg); lane 3, βTC3-neo membranes (20 μg). Shown to the right are the positions of two marker proteins. No other bands were observed in this 5-min exposure.

ob/ob islets, the contribution of RNA from the non-β-cells present in whole islets, as well as from contaminating pancreatic acinar cells, makes it difficult to interpret in terms of specific expression in β-cells (19).

Studied by whole-cell patch clamp techniques, βTC3-neo cells have similar delayed rectifier K⁺ currents to normal rodent or human β-cells (2, 22, 27). Exposure of βTC3-neo cells to 10 mM TEA caused inhibition (>90%) of the outward currents (Fig. 4). TEA blocks a variety of K⁺ channel types over distinct concentration ranges. Most sensitive to TEA are calcium-activated K⁺ channels (K⁺o, 0.14 mM), followed by delayed rectifier K⁺ channels (K⁺d, 1.4 mM) and K-ATP (K⁺p, 22 mM) (2, 14, 27). The K⁺o for block was estimated to be 0.8 mM, approximating the reported Kd for block by TEA of Kv3.2 and 2.1 channels. Similarly, the reported Kd for 4-AP block of Kv2.1 and Kv3.2 in the low millimolar range (30, 31) are consistent with our experimental observations. By contrast, most Kv1 channels of the type encoding β-cell-like delayed rectifier K⁺ currents (Kv1.1–3, 1.5–7) are not sensitive to low millimolar concentrations of TEA (with the exception of Kv1.1 and 1.6) (19, 32). Further-
The addition of 20 mM TEA induced a transient rise in 
\([Ca^{2+}]_i\), followed by rapid oscillations, again similar to the responses seen with bTC3-neocells (cf. Fig. 5A). Likewise, in mouse islets TEA augmented the 
\([Ca^{2+}]_i\) oscillations induced by glucose, in agreement with previous results on the potentiating effects of TEA on mouse islet electrical activity (Fig. 6B) (17).

The 
\([Ca^{2+}]_i\) oscillations induced by K\(^+\) channel block were immediately abolished by exposure to 500 nM nitrendipine, indicating their dependence on Ca\(^{2+}\) influx through L-type calcium channels (34). To confirm that the induction by TEA of glucose-stimulated 
\([Ca^{2+}]_i\) oscillations was in fact due to facilitating depolarization-dependent increases in Ca\(^{2+}\) influx, we carried out simultaneous measurements of membrane potential and 
\([Ca^{2+}]_i\) in single bTC3-neo cells. Application of 1 mM glucose caused a 20-mV depolarization and low amplitude spike activity (Fig. 7). Addition of 20 mM TEA caused a further abrupt depolarization followed by sinusoidal oscillations of membrane potential that were perfectly synchronized with the oscillations in 
\([Ca^{2+}]_i\), (Fig. 7). The oscillations, although small in magnitude, were centered around 0 mV, the peak membrane potential for Ca\(^{2+}\) permeation through L-type Ca\(^{2+}\) channels (2). Thus, it is likely that the oscillations in 
\([Ca^{2+}]_i\), induced by TEA stem from augmentation of membrane potential oscilla-

**FIG. 2.** Alignments of K\(^+\) channel sequences amplified from bTC3 cells. A, alignment of the first 147 bp of plasmid insert mp17 obtained from lane 1 in Fig. 1A, with mouse Kv2.1 (mshab, GenBank accession no. M64228). B, alignment of the first 306 bp from mp9111, a Kv3.2-like cDNA obtained from lane 2 in Fig. 1A, with a rat Kv3.2 sequence (RKSHIIIA, Genbank accession no. M34052; a mouse Kv3.2 sequence has not been previously reported) showing 95.8% identity over 306 bp. The deduced peptide sequence is 99% identical to rat Kv3.2 (RKSHIIIA) in this region.

**FIG. 3.** Amplification of K\(^+\) channel cDNAs in mouse and rat FACS-purified b-cells. A, detection of Kv2.1 in mouse b-cells. RT-PCR was performed with the following primer pairs: lane 1, DNA standards; lane 2, Kv2.1 primers, mouse b-cell cDNA; lane 3, Kv2.1 primers, no template; lane 4, Kv4.1 primers, mouse b-cell cDNA; lane 5, Kv4.1 primers, no template; lane 6, proinsulin primers, mouse b-cell cDNA; lane 7, DNA standards. B, absence of Kv1.1 and Kv1.3 in purified rat b-cells. Lane 1, DNA standards; lane 2, Kv1.1 primer, rat brain cDNA; lane 3, rat b-cell cDNA, Kv1.1 primers; lane 4, Kv1.1 primers, no template; lane 5, Kv1.3 primers, rat brain cDNA; lane 6, Kv1.3 primers, water control; lane 8, proinsulin primers, rat b-cell cDNA; lane 9, DNA standards. C, detection of Kv2.1 in rat b-cells. Lane 1, DNA standards; lane 2, Kv2.1 primers, rat b-cell cDNA; lane 3, Kv2.1 primers, no template; lane 4, proinsulin primers, mouse b-cell cDNA; lane 5, DNA standards.

**FIG. 4.** TEA and 4-AP cause parallel block of delayed rectifier K\(^+\) currents and stimulation of insulin secretion. Upper panels, families of outward currents recorded from single bTC3-neo cells in the absence (upper panels) and presence of 1 mM TEA or 4-AP (middle panels). In whole-cell clamp configuration, the membrane potential was clamped in 20-mV increments from −60 to +80 mV from a holding potential of −60 mV. Same cell in both panels is shown before and after drug application (representative of five separate experiments). Lower panels: dose-response curves showing percentage block of outward delayed rectifier K\(^+\) current (measured during the clamp pulse to +40 mV after leak subtraction) and insulin secretion as a function of drug concentration.
Insulin secretion was also stimulated by 4-AP in the initial small reduction in $[\text{Ca}^{2+}]_i$ monophasic rise following a lag period of approximately 300 s. Note the initial small reduction in $[\text{Ca}^{2+}]_i$, which was a consistent finding. Higher concentrations of glucose had no consistent further effect on $[\text{Ca}^{2+}]_i$, as has been reported for the maximal glucose responsiveness of these cells for insulin secretion. Application of 20 mM TEA in the continued presence of glucose initiated large amplitude $[\text{Ca}^{2+}]_i$ oscillations. Representative of more than 50 experiments. Glucose dependence of the TEA-activated $[\text{Ca}^{2+}]_i$ oscillations. In the continued presence of 20 mM TEA, the glucose concentration was varied from 1 to 0.01 mM which caused a progressive decrease in the frequency of the $[\text{Ca}^{2+}]_i$ oscillations. In the absence of glucose, TEA did not induce $[\text{Ca}^{2+}]_i$ oscillations (34).

**Effect of TEA concentration on glucose-dependent $[\text{Ca}^{2+}]_i$ alterations**

| TEA (mM) | n | Increase in $[\text{Ca}^{2+}]_i$ | $[\text{Ca}^{2+}]_i$ oscillations |
|----------|---|-------------------------------|---------------------------------|
| 1        | 55 | 55                            | 11                              |
| 5        | 116 | 64                            | 41                              |
| 10       | 86  | 85                            | 72                              |
| 20       | 32  | 100                           | 88                              |

a Percentage of cells that display an increase in $[\text{Ca}^{2+}]_i$, with TEA.

b Percentage of cells that demonstrate an oscillatory pattern of increased $[\text{Ca}^{2+}]_i$.

...as a result of TEA-induced modulation of a K$^+$ channel conductance.

Finally, the effects of delayed rectifier K$^+$ current block on stimulating glucose-dependent insulin secretion were examined. In mouse islets, 20 mM TEA augments glucose-stimulated insulin secretion, with a threshold of about 2 mM (17). In the $\beta$TC3-neo cells, TEA produced a dose-dependent stimulation of insulin secretion with a threshold of about 1 mM, and 20 mM TEA induced a 4-fold increase in insulin release compared with glucose alone (Fig. 4). TEA in the absence of glucose had no effect on insulin secretion, in agreement with its secretagogue effects depending on block of delayed rectifier K$^+$ channels alone (34). Insulin secretion was also stimulated by 4-AP in the presence of glucose over the same concentration range (3–10 mM) where suppression of delayed rectifier K$^+$ currents occurred in $\beta$TC3-neo cells (Fig. 4). It is important to consider possible interactions of TEA with other membrane proteins. Although TEA can block K-ATP, the concentrations required for complete block are much higher than used here, and K-ATP is already substantially blocked in extracellular solutions with elevated glucose concentrations (data not shown). TEA has also been reported to block Cl$^-$ channels (35). However, the higher concentrations required ($K_c$ 11.8 mM, with a maximum block of only about 60% at 60 mM (35) cannot account for the dramatic stimulation of signal transduction shown (Figs. 5–7). Furthermore, 4-AP, which produced effects similar to TEA on insulin secretion, has no effect on Cl$^-$ channels (35). In fact, several studies have shown that Cl$^-$ channel blockers actually inhibit, rather than stimulate, insulin secretion (36, 37).

Our results confirm and extend previous observations on the importance of delayed rectifier K$^+$ channels in repolarization of the $\beta$-cell plasma membrane and implicate specific delayed rectifier genes in this process. We report for the first time the expression in rat and mouse $\beta$-cells of Kv2.1 and in $\beta$TC3-neo insulinoma cells of Kv2.1 (Shab)- and Kv3.2 (Shaw)-related transcripts. The characteristics of the $\beta$-cell delayed rectifiers are in fact most consistent with those of Kv2.1 currents, as opposed to other known delayed rectifier genes. These findings extend the distribution of the Kv2.1 channel to hormone-secreting cells, in addition to striated muscle and neurons (19). The dose-response relationships for TEA- and 4-AP-stimulated insulin secretion correlate directly with the block of Kv2.1-like voltage-dependent outward K$^+$ currents, as do the effects of TEA on glucose-dependent $[\text{Ca}^{2+}]_i$, oscillations. It is likely, therefore, that the observed oscillations in membrane potential and $[\text{Ca}^{2+}]_i$, in $\beta$TC3 cells induced by TEA and 4-AP stem directly from block of voltage-dependent K$^+$ channels. Furthermore, our observations in rodent islets where similar induction of $[\text{Ca}^{2+}]_i$ oscillations was observed point to the importance of delayed rectifier K$^+$ channels in normal $\beta$-cells as well.

Last, our studies extend previous observations on the importance of delayed rectifier K$^+$ channels in repolarization of the $\beta$-cell plasma membrane and implicate specific delayed rectifier genes in this process.
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FIG. 7. TEA stimulates simultaneous oscillations in membrane potential and [Ca2+]i. A, simultaneous recording of membrane potential (upper trace) and [Ca2+]i (lower trace) in a single bTC3-neo cell. [Ca2+]i was estimated using fura-2 fluorescence while the membrane potential was monitored using perforated patch techniques. Application of 1 mM glucose (solid bar) caused a coincident membrane potential and increase in [Ca2+]i. Application of 10 mM TEA in the continued presence of glucose caused a further increase in [Ca2+]i, followed by a train of oscillations. In parallel, low amplitude oscillations in membrane potential translate into large changes in [Ca2+]i, reflecting the steep voltage dependence of L-type calcium channel activation.

tgether these studies have also revealed an important insight into the underlying mechanism regulating b-cell oscillations: essentially all of the outward K+ currents have to be suppressed in order to unmask the endogenous b-cell oscillator, indicating that the changes in conductance(s) underlying this mechanism could be very small.