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Response to ADP-Ribose by Activation of TRPM2 in the CRI-G1 Insulinoma Cell Line

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Abstract. The response to intracellular ADP-ribose in the rat CRI-G1 insulinoma cell line was studied using a patch-clamp method. Dialysis of ADP-ribose into cells induced a response in a dose-dependent manner. The reversal potentials in various solutions showed that the ADP-ribose-gated channel was a Ca^{2+}-permeable nonselective cation channel. In inside-out recordings, ADP-ribose and β-NAD induced responses in the same patch. The single-channel current-voltage relationships for ADP-ribose- and β-NAD-induced responses were almost identical, indicating that ADP-ribose and β-NAD activated the same channel. The physiological properties of the ADP-ribose-gated channel are similar to those we reported previously for the cloned transient receptor potential channel TRPM2. Moreover, RT-PCR analysis showed that TRPM2 was abundantly expressed in CRI-G1 cells, suggesting that the ADP-ribose-gated channel represents the native TRPM2 channel in CRI-G1 cells. These results suggest that ADP-ribose can be an endogenous modulator of Ca^{2+} influx through the TRPM2 channel into CRI-G1 cells.

Key words: ADP-ribose — β-NAD — TRPM2 — CRI-G1 cell — Calcium

Introduction

Elevation of the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is a significant process in β cells for insulin secretion. Metabolism of glucose leads to an increase in the cytosolic ATP/ADP ratio, closure of ATP-dependent K^+ channels in the plasma membrane, and thus depolarization of the β cell (Efendic, Kindmark & Berggren, 1991; Berggren & Larsson, 1994). This results in Ca^{2+} influx through voltage-gated L-type Ca^{2+} channels, an increase in $[Ca^{2+}]_i$, and, thereby, release of insulin. In addition to this mechanism, it was reported that inositol 1,4,5-trisphosphate (IP_3) and cyclic ADP-ribose (cADPR) are involved in increases in $[Ca^{2+}]_i$ from intracellular stores in β cells. IP_3 was shown to mobilize $[Ca^{2+}]_i$ in permeabilized insulin-secreting cells (Biden et al., 1984), and cADPR also increased $[Ca^{2+}]_i$ in pancreatic β cells (Takasawa et al., 1993a, 1998; Okamoto, Takasawa & Nata, 1997). As to cADPR, however, it is unclear whether cADPR is involved in Ca^{2+} mobilization and insulin secretion in β cells since the increases in $[Ca^{2+}]_i$ were not confirmed in several experiments (Islam, Larsson & Berggren, 1993a, 1993b; Rutter et al., 1994; Willmott, Galione & Smith, 1995; Webb et al., 1996). But $[Ca^{2+}]_i$ mobilization pathways from intracellular Ca^{2+} stores were also reported to be important for insulin secretion (Rojas et al., 1994). Thus, Ca^{2+} mobilization is a significant trigger for insulin secretion in β cells.

In addition to insulin secretion, however, overloading of intracellular Ca^{2+} in β cells is also linked to cell death (Kaiser & Edelman, 1977; McConkey et al., 1990; Takei & Endo, 1994). Hence, it is important to elucidate all of the Ca^{2+} mobilization pathways in β cells for a better understanding of cell functions.

Recently, it was reported that members of the transient receptor potential (TRP) channel superfamily may represent additional Ca^{2+} influx components (Putney & McKay, 1999; Harteneck, Plant & Schultz, 2000; Hofmann et al., 2000; Missiaen et al., 2000). Several reports revealed that TRPM2, which was designated TRPC7 (Nagamine et al., 1998) or LTRPC2 (Harteneck et al., 2000) but was recently named TRPM2 according to Montell et al. (2002), a member of the TRP superfamily, formed a Ca^{2+}-permeable

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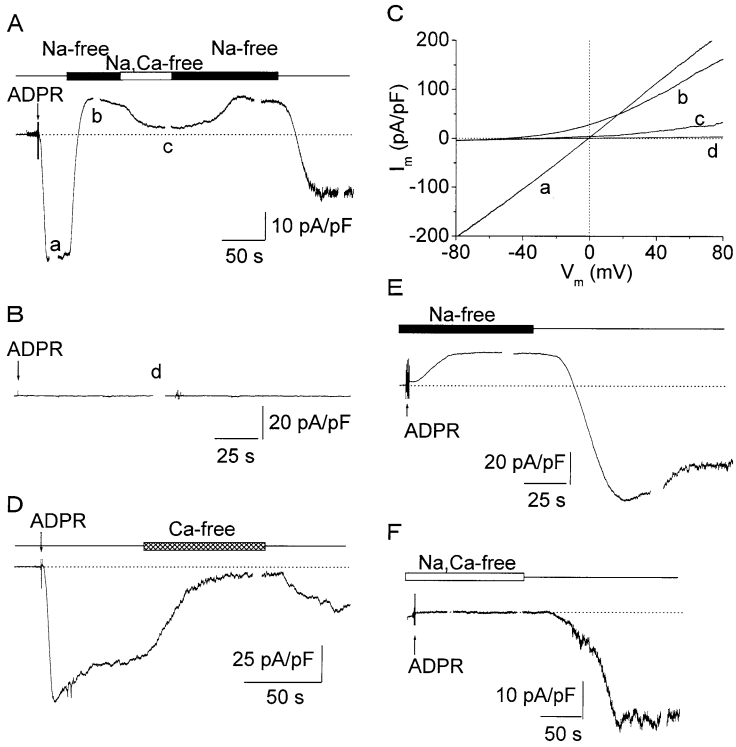

Fig. 1. Whole-cell current responses to ADPR dialyzed into CRI-G1 cells. (*A*) Typical response induced by intracellular dialysis of 0.5 mM ADPR from the patch pipette to CRI-G1 cells. The holding potential was −20 mV. Black and white bars above the current trace indicate cell superfusion with Na^+-free and Na^+, Ca^{2+}-free solution, respectively. The thin continuous line indicates superfusion with normal solution (standard condition). The dotted line indicates the zero-current level. (*B*) When the pipette was filled with a ADPR-free internal solution, the CRI-G1 cells held a steady baseline after membrane rupture during the test-period of 3 min. (*C*) Whole-cell *I-V* relationships for the current evoked by 0.5 mM ADPR. Current was measured by applying a voltage ramp (50 mV/sec) from −80 to +80 mV in the normal extracellular (*a*), Na^+-free (*b*) and Na^+, Ca^{2+}-free solution (*c*) shown in *A*. (*d*) Control ramp current shown in *Bd*. (*D*) Effect of extracellular Ca^{2+} on the ADPR-induced current response. Cross-hatched bar above the trace indicates superfusion with Ca^{2+}-free solution. (*E*) Intracellular dialysis of 0.5 mM ADPR from the patch pipette into the CRI-G1 cells in Na^+-free solution evoked an outward current at a holding potential of −20 mV. (*F*) Intracellular dialysis of 0.5 mM ADPR from the patch pipette in Na^+, Ca^{2+}-free solution did not induce a current response at a holding potential of −20 mV. Upon change to normal solution, an inward current was gradually induced.

nonselective cation channel that was activated by intracellular ADP-ribose (ADPR) and β-NAD (Perraud et al., 2001; Sano et al., 2001; Hara et al., 2002). Furthermore, it was reported that ADPR directly gated the fertilization channels of ascidian oocytes (Wilding et al., 1998). It is possible that ADPR serves as a second messenger for Ca^{2+} influx in β cells. In the present study, we investigated whether ADPR induces a response and whether TRPM2 is expressed in CRI-G1 cells (a rat insulin-secreting cell line).

Materials and Methods

Cell Culture

CRI-G1 cells were obtained from ECACC (Salisbury, UK; ECACC no. 87052701) and cultured in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (JRH BIOSCIENCES, Lenexa, KS) in a humidified atmosphere of 5% CO_2 in air at 37°C. Cells were passed at 3- or 4-day intervals and used 1–3 days after plating.

Electrophysiological Studies

Voltage clamp measurements were performed as described previously (Hamill et al., 1981). Recordings were made with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA), using patch electrodes with a resistance of 3–10 MΩ. Single-channel data were filtered at 1 kHz and sampled at 20 kHz. The normal internal pipette solution contained (in mM) 150 CsCl, 5 $MgCl_2$, 10 HEPES, pH 7.2 (adjusted with CsOH). The normal external solution contained (in mM) 145 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, pH 7.4 (adjusted with NaOH). The 0.2 mM-Ca^{2+} pipette solution contained (in mM) 140 CsCl, 0.6 $MgCl_2$, 5.2 $CaCl_2$, 5 EGTA, 10 HEPES, pH 7.2 (adjusted with CsOH), and the 0-Ca^{2+} pipette solution contained (in mM) 140 CsCl, 0.6 $MgCl_2$, 5 EGTA, 10 HEPES, pH 7.2 (adjusted with CsOH). The Na^+-free solution contained (in mM) 150 *N*-methyl-D-glucamine, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, pH 7.4 (adjusted with HCl). The (nominally) Ca^{2+}-free solution contained (in mM) 145 NaCl, 5 KCl, 2 $MgCl_2$, 10 HEPES, pH 7.4 (adjusted with NaOH). The Na^+, Ca^{2+}-free solution contained (in mM) 150 *N*-methyl-D-glucamine, 2 $MgCl_2$, 10 HEPES, pH 7.4 (adjusted with HCl). For the inside-out patch experiments, the recording pipette contained the normal external solution, and the bath solution contained (in mM) 150 KCl, 2 $MgCl_2$, 10 HEPES, pH 7.4 (adjusted with KOH). All recordings were performed at room temperature (25°C) within 30 minutes after transfer to the recording chamber. All values are expressed as the mean ± SEM. Analysis was carried out on a personal computer, using pCLAMP6 and Clampfit8 software (Axon Instruments). Statistical significance was tested using Student's *t*-test. No corrections were made for junction potentials. All reagents were purchased from Sigma (St. Louis, MO).

Molecular Cloning of Rat TRPM2

The polymerase chain reaction (PCR) was carried out with rat brain cDNA (CLONTECH, Palo Alto, CA, USA). A primer set was designed from human TRPM2 CDS (Genbank accession number: AB001535): 5′-GGGAAGGCAAGGATGGTC-3′; 5′-TCCTTCATCATCCGCTTCAC-3′. The PCR reaction was performed for 35 cycles at 98°C for 15 sec, 60°C for 30 sec, and 72°C for 3 min. Amplified PCR products were subcloned into pCR-TOPO (Invitrogen, Carlsbad, CA, USA) and sequenced for both strands.

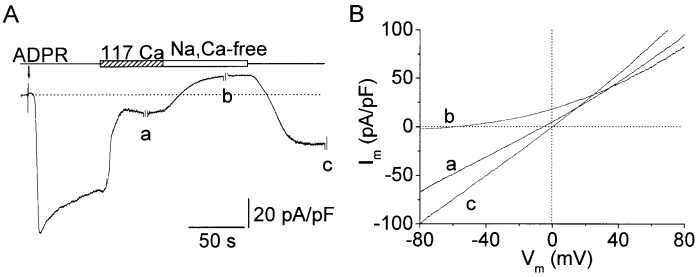

Fig. 2. Ca^{2+} permeability of the ADPR-gated channel. (*A*) The response to 0.5 mM ADPR in isotonic 117 mM Ca^{2+} solution. The holding potential was −20 mV. Bars above the trace indicate replacement of solutions. (*B*) The *I-V* relationship for currents shown in *A* measured by applying a voltage ramp (50 mV/sec) from −80 to +80 mV in 117 mM Ca^{2+} (*a*), Na^+, Ca^{2+}-free (*b*) and normal extracellular solution (*c*).

RT-PCR Analysis

Total RNA of the CRI-G1 cells was isolated with Isogen (TOYOBO, Osaka, Japan), and rat brain poly(A)$^+$ RNA (CLONTECH) was reverse-transcribed to generate first-strand cDNAs with Superscript II (Invitrogen). PCR was performed with these first-strand cDNAs by using the above primer set for 35 cycles at 98°C for 15 sec, 61°C for 30 sec and 72°C for 2 min. The amplified fragments were subcloned into pCR-TOPO and sequenced for both strands. As a control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified using specific primers (CLONTECH).

Results

Whole-Cell Current Induced by Intracellular Dialysis of ADPR

Intracellular dialysis of 0.5 mM ADPR from the patch pipette to the CRI-G1 cells evoked an inward current in all cells examined at a holding potential of −20 mV (Fig. 1*A*). The current diminished gradually. Removal of Na^+ from the extracellular solution caused an outward current and continuous absence of Ca^{2+} caused suppression of the response. Return to Ca^{2+}-containing Na^+-free solution, restored the outward current. Return to the normal (Ca^{2+}- and Na^+-containing) solution reversed to an inward current, but of decreased ampltitude compared to the initial response. No such currents were observed in CRI-G1 cells over the test interval for 5 min after membrane rupture without ADPR in the pipet (Fig. 1*B* and *C*). As shown in Fig. 1*C*, the current-voltage (I-V) relationship was examined by applying a voltage ramp during the response in the normal, Na^+-free or Na^+, Ca^{2+}-free solution. The slope of the I-V curve measured during the response in the normal solution was almost linear, indicating that the response lacks voltage dependency. The mean reversal potential was estimated to be 0.6 ± 0.5 mV (n = 10). In the Na^+-free solution, the mean reversal potential of the responses to ADPR was estimated to be −50.6 ± 1.2 mV (n = 5). In the Na^+, Ca^{2+}-free solution, however, both inward and outward currents were inhibited, suggesting that the inhibition of the current was probably caused by removal of the extracellular Ca^{2+}. In fact, the magnitude of the inward current induced by ADPR was decreased in the extracellular Ca^{2+}-free solution (Fig. 1*D*). In addition, dialysis of ADPR from the patch pipette in the Na^+-free solution evoked an outward current at a holding potential of −20 mV (n = 4; Fig. 1*E*), whereas a response to ADPR was never observed in the Na^+, Ca^{2+}-free solution (n = 4; Fig. 1*F*). These results suggest that external Ca^{2+} is required to evoke the response.

The permeability of the ADPR-gated channel to Ca^{2+} was investigated. Replacement of the bath solution with isotonic 117 mM $CaCl_2$ solution decreased the ADPR-induced current (Fig. 2*A*). Change to the Na^+, Ca^{2+}-free solution, the current reversed to an outward current (Fig. 2*A*). However, it should be noted that the response to ADPR was not inhibited in the Na^+, Ca^{2+}-free solution. This may be due to incomplete elimination of Ca^{2+} from the extracellular solution. The reversal potential of the ADPR-induced response in the 117 mM $CaCl_2$ solution slightly shifted to a negative potential compared with that in the normal solution (Fig. 2*B*). The mean value was −5.7 ± 1.4 mV (n = 6), and the permeability ratio was 0.54 for P_{Ca}/P_{Cs} when calculated with the Goldman-Hodgkin-Katz modified constant-field equation (Lewis, 1979). These results suggest that the ADPR-gated channel in CRI-G1 cells is a Ca^{2+}-permeable nonselective cation channel.

Characteristics of the ADPR-Induced Response

The magnitude of the inward current induced by ADPR increased as the ADPR concentration was increased (Fig. 3*A*). Figure 3*B* shows the magnitude of the response induced by ADPR plotted as a function of the ADPR concentration. The current first appeared at a concentration of 50 μM and increased with increasing ADPR concentration, reaching a plateau at 500 μM. Data points were fitted by the Hill equation, giving a concentration of 90 μM for half-maximal activation and a Hill coefficient of 2.4.

The effect of $[Ca^{2+}]_i$ on the ADPR-induced response was also examined. Using the pipette solution containing 0.2 mM free Ca^{2+}, the current density of the ADPR-induced current was 44.5 ± 5.1 pA/pF (n = 7). However, decreasing the free Ca^{2+} concentration in the pipette solution to low nanomolar levels with 5 mM EGTA and no added Ca^{2+}, the current density of the ADPR-induced current was significantly

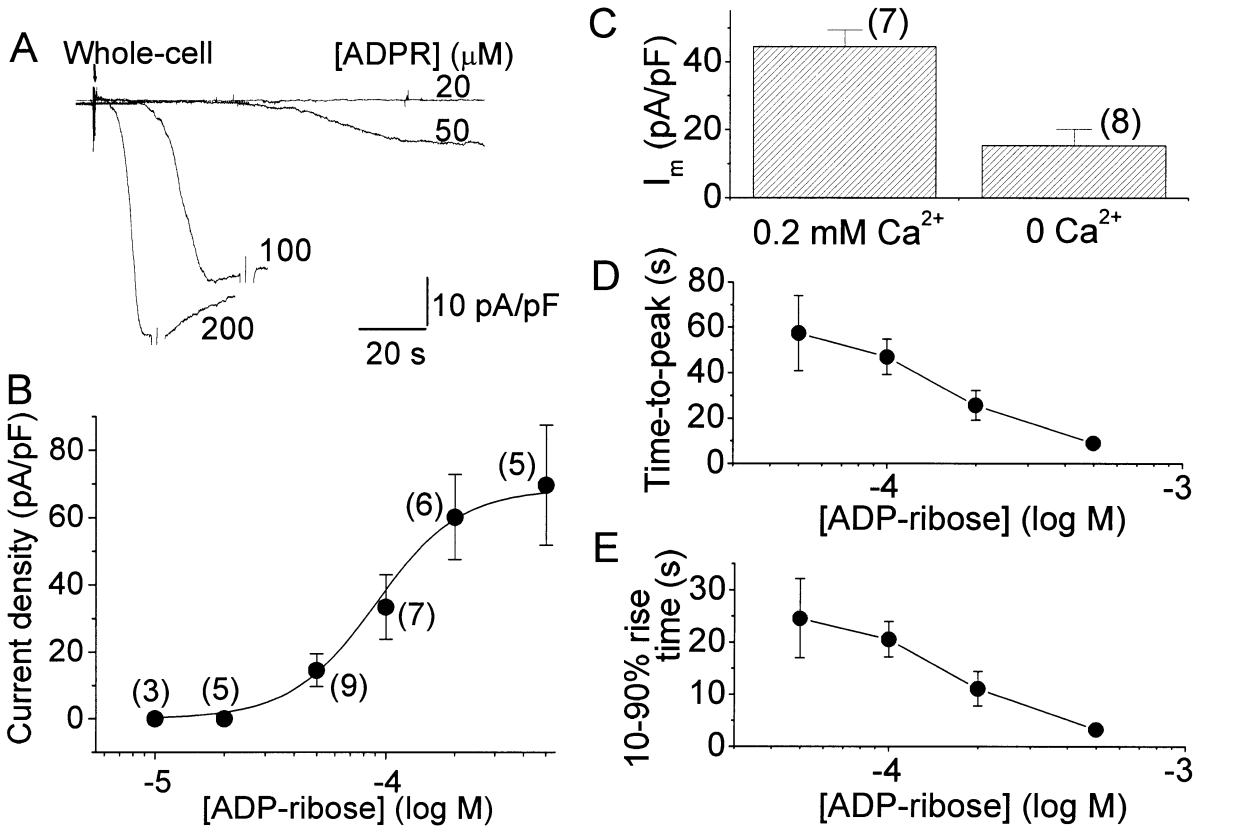

Fig. 3. Properties of the responses to ADPR. (*A*) Responses induced by intracellular dialysis of ADPR from the patch pipette to CRI-G1 cells. The concentrations of ADPR contained in the pipette are shown for each trace. The holding potential was −20 mV. (*B*) Dose dependence of the response induced by intracellular application of ADPR. The data were fitted by the Hill equation with $n = 2.4$, concentration for half-maximal activation ($K_{1/2}$) = 90 μM, and maximal current density (I_{max}) = 69 pA/pF. Each point is the mean ± SEM of data obtained from n preparations indicated in parentheses. (*C*) Mean current densities of ADPR-induced responses with Ca^{2+} (0.2 mM Ca^{2+}) or without Ca^{2+} (0 Ca^{2+}) in the patch pipette solution. The average current densities between these pipette solutions were significantly different when the data were analyzed by Student's t-test ($p < 0.01$). The numbers in parentheses show the number of tested cells. (*D*) The time interval between 10 and 90% of the peak value of ADPR-induced responses plotted as a function of the ADPR concentration. (*E*) The time-to-peak value of ADPR-induced responses plotted as a function of the ADPR concentration.

reduced (15.4 ± 4.9 pA/pF, $n = 8$; Fig. 3*C*). This result suggests that Ca^{2+} must be present on the cytoplasmic side of the membrane for channel activation.

As shown in Fig. 3*D*, which assesses the kinetics of the ADPR-induced inward current, the 10–90% rise times (the time interval between 10 and 90% of the peak response) were plotted as a function of the ADPR concentration. The 10–90% rise time became significantly shorter as the ADPR concentration increased. Also, the time-to-peak value upon membrane rupture increased as the ADPR concentration decreased (Fig. 3*E*).

Single-Channel Currents Activated by ADPR and β-NAD Recorded in Inside-out Mode

In inside-out patch recordings, application of ADPR to the bath solution induced multi-channel currents instantaneously and reversibly (Fig. 4*A*). Figure 4*B* shows single-channel currents activated by ADPR at different potentials. The single-channel I-V relationship for the channel activated by ADPR was linear with a unitary conductance of 83 pS, and it reversed at 0 mV (Fig. 4*C*, *circles*). These results suggest that ADPR directly caused the channel opening.

A previous report showed that a β-NAD-activated channel exists in the plasma membrane of CRI-G1 cells (Herson, Dulock & Ashford, 1997). Indeed, β-NAD (1 mM) evoked a response in an inside-out patch membrane that responded to ADPR (Fig. 4*A*). We also observed that application of β-NAD to membrane patches that responded to ADPR always evoked a response, but a response was never observed in membrane patches that did not respond to ADPR. The properties of the single-channel I-V relationship for the β-NAD-induced response are close to those for the ADPR-induced response (Fig. 4*C*, *triangles*). These results indicate that ADPR and β-NAD directly activate the same channel in the plasma membrane of CRI-G1 cells.

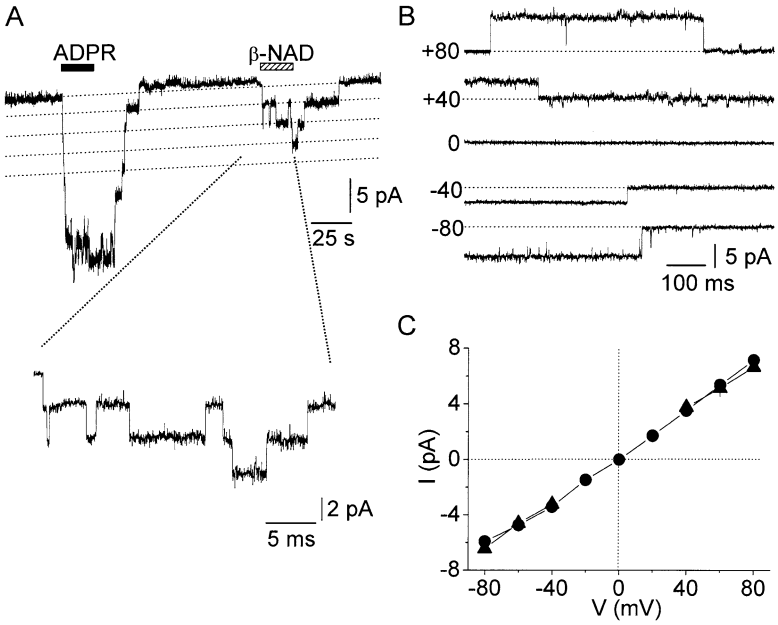

Fig. 4. Characteristics of single-channel currents induced by application of ADPR and β-NAD in inside-out patches. (*A*) Multi-channel currents activated by ADPR (0.5 mM) and β-NAD (1 mM) in an inside-out patch excised from CRI-G1 cells. The holding potential was −40 mV. The recording pipette contained the normal external solution, and the bath solution contained 150 mM KCl, 2 mM $MgCl_2$. The bars above the trace indicate the periods of stimulation. (*B*) Single-channel currents activated by ADPR at different membrane potentials obtained in inside-out patches. The dotted lines denote the closed state. (*C*) Single-channel *I-V* relationships activated by ADPR (*circles*) and β-NAD (*triangles*). Linear regression analysis yielded a slope conductance of 83 pS.

Expression of Rat TRPM2 in CRI-G1 Cells

We recently reported that human TRPM2 formed a Ca^{2+}-permeable nonselective cation channel that was activated by intracellular ADPR and β-NAD (Sano et al., 2001). In order to identify rat TRPM2, a PCR experiment was carried out on rat brain cDNA, in which TRPM2 is abundantly expressed, by using a human TRPM2-specific primer set. The deduced amino-acid sequence showed 88.3% identity with human TRPM2 by the clustal method and shared 20.6%, 37.1%, and 21.4% identity with MLSN1 (Prawitt et al., 2000), MTR1 (Duncan et al., 1998) and TRP-PLIK (Runnels, Yue & Clapham, 2001), respectively (Fig. 5*A*).

To identify rat TRPM2 expressed in CRI-G1 cells, RT-PCR analysis was performed on total RNA from CRI-G1 cells. PCR was carried out under highly stringent conditions with the above primer set. An amplified product of the expected size was obtained (Fig. 5*B*), and its identity was confirmed by its DNA sequence. This result indicates that rat TRPM2 is endogenously expressed in CRI-G1 cells.

Discussion

In this paper, we have shown that dialysis of ADPR induced an inward current in CRI-G1 cells. The ADPR-induced response was dose-dependent. We also demonstrated that the ADPR-gated channel is a Ca^{2+}-permeable nonselective cation channel that is located in the plasma membrane of CRI-G1 cells and is modulated by intracellular and extracellular Ca^{2+}. It has been reported that ADPR activated the fertilization channel of ascidian oocytes (Wilding et al., 1998), and ADPR is thought to be responsible for the production of fertilization current. Therefore, it was suggested that ADPR might serve as a second messenger for Ca^{2+} influx in CRI-G1 cells.

The ADPR-gated channel also responded directly to β-NAD. Recently, it was reported that the TRPM2 channel, a member of the TRP superfamily, formed a Ca^{2+}-permeable nonselective cation channel and responded to both ADPR and β-NAD (Perraud et al., 2001; Sano et al., 2001; Hara et al., 2002). The biophysical properties of the ADPR-gated channel in CRI-G1 cells closely resemble those of the TRPM2 channel. The concentration for half-maximal activation of the ADPR-gated and the TRPM2 channel was 90 and 70 μM, single-channel conductance was 83 and 76 pS, and Ca^{2+} permeability ratio was 0.54 and 0.67, respectively, and activation of both channels is Ca^{2+} dependent (Perraud et al., 2001). As shown in the present study, TRPM2 is expressed endogenously in CRI-G1 cells. These results suggest, therefore, that the responses to ADPR are mediated via the activation of functionally expressed TRPM2.

It has previously been suggested that in rat pancreatic β cells, cADPR is synthesized in response to glucose (Takasawa et al., 1993b), which subsequently mobilizes intracellular Ca^{2+}, which, in turn, stimulates insulin secretion. Moreover, the formation of ADPR was observed through cADPR hydrolysis mediated via CD38 (Takasawa et al., 1993b). Subsequent studies have, however, demonstrated that cADPR is ineffective in releasing intracellular Ca^{2+} from permeabilized rat pancreatic islet (Rutter et al., 1994) or mouse β cells (Islam et al., 1993a), and from the insulin-secreting cell lines INS-1 (Rutter et al., 1994) or RINm5F (Islam et al., 1993a). Therefore, it remains unclear whether cADPR is involved in Ca^{2+} mobilization and insulin

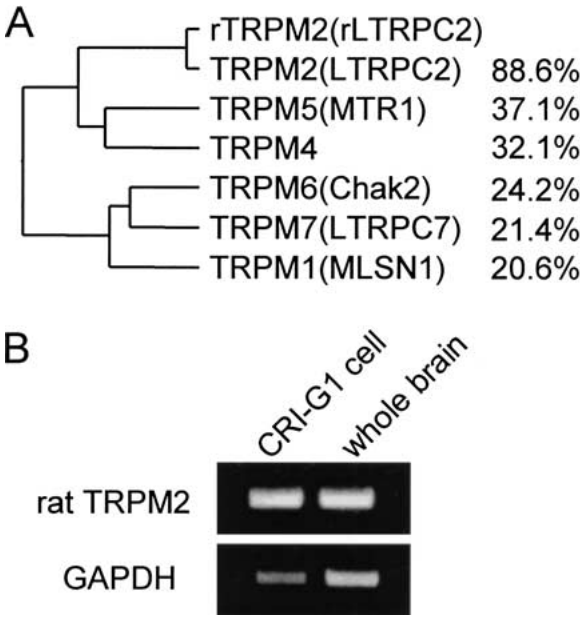

Fig. 5. RT-PCR analysis of CRI-G1 cells. (*A*) Proposed phylogenetic tree of rat TRPM2 and members of LTRPC family. The evolutionary tree was calculated by the clustal method. The evolutionary distance is shown as the total branch length. The percent values indicate the pair distance of rat TRPM2. (*B*) Expression of rat TRPM2 in CRI-G1 cells. Total RNA from CRI-G1 cells was subjected to RT-PCR, and then the 1529 bp product was amplified. As positive control for these RT-PCR reactions, GAPDH was amplified.

secretion in β cells. In this report, we showed that ADPR could activate the insulin-secreting cell. Hence, it is possible that ADPR may function as a Ca^{2+}-mobilization factor instead of cADPR in vivo.

A previous report showed that a Ca^{2+}-permeable nonselective cation channel activated by β-NAD (NS_{NAD} channel) is present in the plasma membrane of CRI-G1 cells (Herson, Dulock & Ashford, 1997). Interestingly, the nonselective, NAD-dependent cation channel NS_{NAD} also responded to H_2O_2 (Herson & Ashford, 1997; Herson et al., 1999). Like the NS_{NAD} channel, TRPM2 was not only gated by intracellular ADPR and β-NAD but also activated by H_2O_2 (Hara et al., 2002), and the biophysical properties of the NS_{NAD} channel are similar to those of the TRPM2 channel. Therefore, it is likely that the NS_{NAD} channel is identical to TRPM2.

The properties of TRPM2 were that monovalent cations were almost equally permeable and permeability of Ca^{2+} was intermediate (Perraud et al., 2001; Sano et al., 2001) compared to other Ca^{2+}-permeable nonselective cation channels (Iino, Ozawa & Tsuzuki, 1990; Vernino et al., 1992; Séguéla et al., 1993; Yau, 1994). Since activation of TRPM2 leads to depolarization of the membrane and to Ca^{2+} influx into cells, it is plausible that TRPM2 is involved in insulin secretion and/or cell death in β cells. In fact, exposure of rat pancreatic islets to H_2O_2 resulted in a transient increase in insulin release at the basal nonstimulatory glucose concentration (Maechler, Jornot & Wollheim, 1999), and H_2O_2-induced cell death was observed in rat insulinoma RIN-5F cells, which endogenously expressed TRPM2 (Hara et al., 2002). Recently, Qian et al. (2002) revealed that TRPM2 is expressed in human islets. Hence, the physiological significance of the TRPM2 channel remains unclear, but an increase in the cytosolic Ca^{2+} concentration mediated by activation of TRPM2 may play an important role in the mechanism of deterioration of pancreatic β cell function in disease.

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