Activation of a Calcium-permeable Cation Channel CD20 Expressed in Balb/c 3T3 Cells by Insulin-like Growth Factor-I*

(Received for publication, August 8, 1996, and in revised form, October 4, 1996)

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CD20 functions as a calcium-permeable cation channel. When expressed in Balb/c 3T3 cells, CD20 accelerates the G₁ progression induced by insulin-like growth factor-I (IGF-I). To further characterize how CD20 modulates the action of IGF-I, we investigated whether the activity of CD20 channel was affected by IGF-I. In quiescent cells expressing CD20, IGF-I increased cytoplasmic free calcium concentration, [Ca²⁺],, which was reversed by the removal of extracellular calcium. In contrast, IGF-I did not increase [Ca²⁺], in cells that did not express CD20. In perforated patch clamp recordings, addition of IGF-I to the bath solution augmented the Ca²⁺ permeability, which was reversed by anti-CD20 antibody. In cell-attached patch, calcium-permeable channel activity with unitary conductance of 7 picoSiemens was detected, which was abolished by anti-CD20 antibody. The single channel activities were markedly enhanced when IGF-I was included in the pipette solution, whereas IGF-I added to the bath solution was ineffective. When cells were first exposed to pertussis toxin, activation of the channel by IGF-I was blocked. Transfection of cDNA for Gip2, a constitutive active form of Gαq, activated the CD20 channel. These results indicate that the CD20 channel is regulated by the IGF-I receptor by a mechanism involving pertussis toxin-sensitive G protein.

CD20 is a cell surface protein with a molecular mass of 35 kDa expressed in mature B lymphocytes (1–4). Monoclonal antibodies raised against CD20 affect the growth of B lymphocytes. Thus, most of the antibodies inhibit cell proliferation, whereas some are stimulatory. These observations led to the consideration that CD20 is involved in the regulation of cell growth in lymphocytes. The primary structure of CD20 has been determined by molecular cloning (5–7), and the predicted amino acid sequence indicated that CD20 is a transmembrane protein with four transmembrane domains with both C- and N-terminals located in the cytoplasm. Hence, the structure of CD20 resembles those of ion channels and ion transporters. Indeed, when expressed in fibroblasts, CD20 functions as a calcium-permeable cation channel (8). In lymphocytes, CD20 is phosphorylated by protein kinases including calmodulin-dependent protein kinase. Furthermore, CD20 associates with src family tyrosine kinases including p53/56lck, p56, and p59fyn (9). Since an addition of monoclonal antibody against CD20 induces tyrosine phosphorylation of several proteins (10), CD20 may also participate in the protein tyrosine kinase cascade. Nevertheless, the regulatory mechanism modulating the activity of CD20 is largely unknown and the ligand that activates CD20 has not been identified.

To investigate the function of CD20 as a calcium-permeable channel, we stably expressed CD20 in Balb/c 3T3 fibroblasts (11). CD20 expressed in these cells functioned as a calcium-permeable channel and modulated the growth characteristics of these cells. Thus, CD20 expression accelerated cell cycle progression through the G₁ phase and enabled the cells to progress to the S phase in medium containing low extracellular calcium (11). Insulin-like growth factor-I (IGF-I)1 is a progression factor that induces G₁ progression (12). As described by Stiles et al. (12), IGF-I exerts its action in a cell cycle-dependent manner. When IGF-I is added to quiescent Balb/c 3T3 cells, it cannot induce cell cycle progression (12, 13). In contrast, cells progress toward the S phase in response to IGF-I when they are first exposed to platelet-derived growth factor followed by epidermal growth factor (14). These are referred to as primed competent cells (14, 15). Therefore, IGF-I exerts its progression activity specifically in primed competent, but not quiescent, cells. However, when CD20 is expressed in quiescent Balb/c 3T3 cells expressing CD20, at least some of the cells progress toward S phase in response to IGF-I (11). This result suggests that IGF-I can elicit progression, even in quiescent cells with the aid of CD20, and implies that a CD20-like protein is critical for the progression activity of IGF-I. If so, it is possible that the function of CD20 expressed in Balb/c 3T3 cells is modulated by IGF-I. In the present study, we investigated this notion. The results indicate that IGF-I, by acting on the IGF-I receptor, activates the channel activity of CD20.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human IGF-I was supplied by Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). Na[¹²⁵I] was obtained from ICN Biomedicals (Costa Mesa, CA). [³H]thymidine and [³²P]dCTP were obtained from Dupont NEN. mAb against CD20 (CBL456) was purchased from Cymbus Bioscience Ltd. (Southampton, UK).

Cell Culture—Balb/c 3T3 cells (clone A31) and Raji cells (B lymphoblastoid cell line) were provided by the RIKEN cell bank (Tsukuba, Japan). Balb/c 3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Life Technologies, Inc.). Raji cells were cultured in RPMI 1640 medium containing 10% fetal calf serum. These cells were cultured under humidified conditions of 95% air and 5% CO₂ at 37 °C.

Transfection of cDNA—The inducible CD20 expression vector (CD20-pME4) was stably transfected into Balb/c 3T3 cells by electroporation as described previously (11). CD20 expressing quiescent Balb/c 3T3 cells were obtained by incubating confluent cells in Dulbecco's modified

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1 The abbreviations used are: IGF-I, insulin-like growth factor-I; [Ca²⁺],, cytoplasmic free calcium concentration; 1V, current-voltage; PTX, pertussis toxin; mAb, monoclonal antibody; MOPS, 4-morpholinepropanesulfonic acid; GTPγS, guanosine 5'-3-O-(thio)triphosphate; GDPβS, guanosine 5'-0-2-(thio)di phosphate.
Eagle’s medium containing 0.5% platelet-poor plasma and 80 μM ZnCl₂ for 24 h. After the treatment with ZnCl₂, all of the cells expressed CD20 (11).

Measurement of DNA Synthesis—DNA synthesis was assayed by measuring [³H]thymidine incorporation into trichloroacetic acid-precipitable DNA as described previously (11). Briefly, cells cultured on glass coverslips were incubated with 2 mm fura-2 acetoxyethyl ester (Dojin Laboratories, Kumamoto, Japan) for 20 min at room temperature (20–26 °C), then placed on a flow-through chamber mounted on the stage of TMD microscope (Nikon, Tokyo, Japan). The perfusion medium contained 137 mM NaCl, 5 mM KCl, 1.25 mM CaCl₂, 5 mM glucose, and 10 mM Hepes/NaOH (pH 7.4). Dual wavelength microfluorometry of the fura-2 fluorescence was performed using CAM-230 (Nikon Bunko, Tokyo, Japan). The emission signals excited at both 340 and 380 nm and the ratio of these signals (340/380 ratio) was recorded. In some experiments, the cytoplasmic free Ca²⁺ concentration was calibrated as described elsewhere (17). Statistical significance was evaluated by analysis of variance.

Electrophysiological Recordings—The perforated-patch (18) and the cell-attached patch clamp techniques were applied for the voltage-clamp studies. Micropipettes were pulled from borosilicate glass capillaries and had tip resistances of around 4 and 8 megohms after filling with a pipette solution. High resolution membrane currents were recorded using an EPC-9 patch-clamp amplifier (HEKA, Lambrecht, Germany) controlled by “E9SCREEN” software on an Atari computer. All voltages were corrected for a liquid junction potential between the bath and pipette solutions. Voltage ramps were of 300-ms duration, covering a range of 20–200 mV. Capacitance and series resistance were canceled before each voltage ramp using the automatic neutralization routine of the EPC-9. For studies using the perforated whole-cell configuration, the micropipettes for electrical recording were filled with a solution containing 143 mM D-glucamine-methanesulfonic acid, 10 mM Ca(OH)₂, and 10 mM HEPES (pH 7.4). The signal was stored on video tape after analogue/digital conversion (Sony PCM 501 ES, modified by Shoshin EM Corp., Okazaki, Japan). For studies using the cell-attached configuration, the micropipette solution (19) was replaced with a solution containing 110 mM BaCl₂ or CaCl₂, 200 nM tetrodotoxin, and 10 mM HEPES (pH 7.4). The total number of functional channels (N) in the patch was estimated by observing the number of peaks detected on the amplitude histogram. As an index of channel activity, N_Po (number of channels multiplied by the open probability) was calculated as

\[ N_Po = \sum_{n=0}^{N} n \cdot P_o \]  

(Eq. 1)

where T is the total recording time, n is the number of channels open, and \( P_o \) is the recording time during which n channels are open. Therefore, \( N_Po \) can be calculated without making assumptions about the total number of channels in a patch or the open probability of a single channel. All electrophysiological experiments were performed at 20–26 °C.

Measurement of Cytoplasmic Free Calcium—The cytoplasmic free Ca²⁺ concentration (\([\text{Ca}^{2+}]_i\)) was monitored using fura-2, as described previously (11). Briefly, cells cultured on glass coverslips were incubated with 2 mm fura-2 acetoxyethyl ester (Dojin Laboratories, Kumamoto, Japan) for 20 min at room temperature (20–26 °C), then placed on a flow-through chamber mounted on the stage of TMD microscope (Nikon, Tokyo, Japan). The perfusion medium contained 137 mM NaCl, 5 mM KCl, 1.25 mM CaCl₂, 5 mM glucose, and 10 mM Hepes/NaOH (pH 7.4). Dual wavelength microfluorometry of the fura-2 fluorescence was performed using CAM-230 (Nikon Bunko, Tokyo, Japan). The emission signals excited at both 340 and 380 nm and the ratio of these signals (340/380 ratio) was recorded. In some experiments, the cytoplasmic free Ca²⁺ concentration was calibrated as described elsewhere (17). Statistical significance was evaluated by analysis of variance.

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Northern Blotting—Cells were harvested, and the total RNA was isolated using Isogene and quantified spectrophotometrically. Total RNA (20 μg) was resolved by electrophoresis on 1.2% agarose gels containing 2.2% formaldehyde, 20 mM MOPS (pH 7.0), 8 mM sodium acetate, and 1 mM EDTA, and transferred to a nylon membrane (Hybond-N⁺, Amersham Corp.), by means of capillary blotting in 10× sodium citrate buffer. Hybridization was performed with a probe labeled with [³²P]dCTP by random priming according to the manufacturer’s instructions (Pharmacia Biotech Inc.). The hybridized membrane was exposed to Kodak XAR film (Eastman Kodak Co.).

RESULTS

Effect of IGF-I on DNA Synthesis in Quiescent CD20-expressing Cells—We showed that IGF-I stimulates DNA synthesis when added to quiescent cells expressing CD20, whereas it has no stimulatory effect on DNA synthesis in untransfected quiescent cells (12). As shown in Fig. 1, IGF-I stimulated [³H]thymidine incorporation in CD20-expressing quiescent cells and the effect of IGF-I was inhibited by a monoclonal antibody (mAb) against CD20 in a dose-dependent manner. This monoclonal antibody also inhibited serum-induced DNA synthesis in Raji cells that express native CD20 (data not shown). It should be noted that mAb against CD20 did not affect DNA synthesis induced by IGF-I (Table I). Furthermore, mAb against CD20 did not cross-react with IGF-I assessed by Western blotting (data not shown).

Effect of IGF-I on [Ca²⁺]ᵢ in CD20-expressing Cells—IGF-I induces oscillatory changes in [Ca²⁺]ᵢ in primed competent cells (17) by activating IGF-operated calcium-permeable channels (15, 20). In quiescent cells, however, IGF-I does not affect [Ca²⁺]ᵢ (18). The effect of IGF-I was inhibited by a monoclonal antibody (mAb) against CD20 in a dose-dependent manner. This monoclonal antibody also inhibited serum-induced DNA synthesis in Raji cells that express native CD20 (data not shown). It should be noted that mAb against CD20 did not affect DNA synthesis induced by IGF-I (Table I). Furthermore, mAb against CD20 did not cross-react with IGF-I assessed by Western blotting (data not shown).

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![](image.png)

FIG. 1. Effect of monoclonal antibody against CD20 on IGF-I-induced DNA synthesis in quiescent Balb/C 3T3 cells expressing CD20. CD20-expressing quiescent cells were incubated for 24 h with (●) or without (○) 1 ng IGF-I in the presence of various concentrations of monoclonal antibody against CD20. [³H]Thymidine incorporation was then measured. Values are the means ± S.E. for four experiments.
The IGF-I-induced elevation of $[\text{Ca}^{2+}]_{c}$ was dependent on extracellular calcium and its removal (Fig. 2A) or the addition of lanthanum (data not shown) abolished the elevated $[\text{Ca}^{2+}]_{c}$. Elevation of $[\text{Ca}^{2+}]_{c}$ induced by IGF-I were completely abolished by the mAb against CD20 and $[\text{Ca}^{2+}]_{c}$ returned to the level of that in cells incubated low-calcium containing medium (6 out of 6 cells) (Fig. 2D). These results suggest that IGF-I stimulated Ca$^{2+}$ influx through the CD20 channel in CD20-expressing cells.

**Effect of IGF-I on the Activity of CD20 Channel in CD20-expressing cells**—Patch-clamp experiments were performed to record the changes in calcium permeability of the membrane induced by IGF-I. We previously reported that IGF-I increases the open probability of a calcium permeable cation channel in primed competent, but not quiescent cells (15, 20). To distinguish CD20 from the native IGF-operated Ca$^{2+}$-permeable cation channel, we used quiescent cells.

First, we examined changes in Ca$^{2+}$ conductance induced by IGF-I using a nystatin-perforated whole-cell patch clamp. The current-voltage (I-V) relationship was obtained by applying voltage ramps from $-100$ to $+100$ mV. Fig. 3A shows a comparison of the I-V curves obtained before and 3 min after the addition of IGF-I. These inward currents were generated by Ca$^{2+}$, since Ca$^{2+}$ is the only cation permeant in this condition. At a holding potential of $-100$ mV, inward current in cells treated with IGF-I was $446 \pm 651\%$ (means $\pm$ S.E., $n=42$) of that in control cells, which was statistically significant ($p$, 0.01). The I-V curve was not changed as a function of time in the absence of IGF-I (Fig. 3B). The Ca$^{2+}$ current was completely abolished by the mAb against CD20 (Fig. 3B) and, in addition, both La$^{3+}$ and Co$^{2+}$ inhibited the Ca$^{2+}$ currents (data not shown). Similar results were obtained when Ca$^{2+}$ was replaced with Ba$^{2+}$ as a charge carrier. When 100 nM insulin was added, the calcium current was similarly enhanced (Fig. 3C). It is notable that we could not identify IGF-I-stimulated Ca$^{2+}$ permeability using the conventional whole-cell patch clamp procedure. This may be due to the fact that, in whole cell configuration, soluble components of the cytosol are quickly dialyzed by the solution filling the pipettes.

Next, we recorded inward currents in cell-attached patches.
Values are the mean ± S.E., n = 8) and extrapolation of the data indicates a reversal potential of +20 mV (Fig. 5). Again, a high concentration of insulin (100 nM) reproduced the effect of IGF-I (data not shown). Barium was used as a charge carrier since calcium ions, there was no significant difference in the IGF-I-induced current. Moreover, current amplitudes were the same whether chloride or aspartate was the anion in the pipette solution, which confirmed that the currents were carried by an influx of cations (Ca\(^{2+}\) or Ba\(^{2+}\)), rather than by an outward anion flux, which would display negative reversal potentials. Potassium cannot be taken into account since this ion was not in the pipette solution and a high concentration of barium inhibits K\(^+\) permeability. No voltage dependence was detected and inhibitors of voltage-dependent calcium channel such as nifedipine and verapamil had no effect on the IGF-I-activated currents.

**Effect of Pertussis Toxin and Mastoparan on the Activity of CD20**—To examine the involvement of pertussis toxin (PTX)-sensitive G protein in IGF-I-induced activation, we studied the effect of IGF-I in PTX-treated cells. When cells were pretreated with PTX (21), the addition of IGF-I in the pipette did not affect the activity of CD20 channel (none of 30 patches) (Fig. 6A). Likewise, IGF-I did not increase calcium current in PTX-treated cells (none of 18 cells) (Fig. 6B). Additionally, IGF-I did not elevate [Ca\(^{2+}\)]\(_i\) in PTX-treated cells (none of 40 cells) (Fig. 6C). Conversely, 50 μM mastoparan, an activator of Gi/Go class of G proteins (22), markedly stimulated the activity of the CD20 channels (43 out of 43 patches) (Fig. 7A) whereas Mas17, an analogue which does not activate the G proteins, was ineffective (none of 14 patches) (Fig. 7B).

**Effect of Transfection of Gip2 on the Activity of the CD20 Channel**—To further assess the role of a G protein in the regulation of CD20 channel, we transfected Balb/c 3T3 cells expressing CD20 with the cDNA for Gip2, a constitutive active...
high concentration of insulin bound to the IGF-I receptor and modulated the activity of CD20. At present, the precise mechanism by which the IGF-I receptor activates calcium-permeable CD20 channels is not certain. The IGF-I receptor structurally resembles the insulin receptor (24–26), and it consists of α- and β-subunits. Ligand binding to the α-subunit results in the activation of the intrinsic tyrosine kinase located in the β-subunit. It is generally accepted that receptor-associated tyrosine kinase is needed for the signal transduction. Yet, the downstream signal leading to the channel activation is not clear at present. Since a single channel current of CD20 was not activated by IGF-I added outside the patch, the regulatory mechanism by which IGF-I receptor activated the channel may be direct, not involving a soluble second messenger. In this regard, previous studies done in our laboratory showed that IGF-I-mediated calcium influx via the calcium-permeable cation channel is blocked by pertussis toxin (27). In addition, IGF-I-mediated calcium entry is blocked by GDPβS and conversely, augmented by GTPγS (28). These results indicated that IGF-I, by acting on the IGF-I receptor, modulates the calcium-permeable channel by a mechanism involving pertussis toxin-sensitive G protein. Similarly, IGF-I stimulates calcium influx in Chinese hamster ovary cells by G_{i2}-dependent mechanism (29), although the precise mechanism by which IGF-I activates the channel via G protein is not yet identified. As shown in Fig. 6A, pertussis toxin also abolished the activation of CD20 by IGF-I. Mastoparan, which directly activates pertussis toxin-sensitive G proteins (22), stimulated the CD20 channels. Additionally, the CD20 channel was markedly activated by the co-expression of cDNA for Gip2, a constitutive active form of G_{i2}. Hence, CD20 can be directly activated by α_{i2} subunit. Taken together, the mechanisms by which IGF-I activates CD20 and the IGF-operated channel may be similar. Recently, Luttrell et al. (30) demonstrated that activation of MAP kinase by IGF-I is attenuated by pertussis toxin. Their results support the notion that a pertussis toxin-sensitive G protein is involved in the signaling system activated by IGF-I. Despite the fact that CD20 is not a natural effector molecule of the IGF-I signaling system, it may provide a good model system with which to study the regulation of the calcium-permeable channel by the IGF-I receptor. Further study is needed to elucidate the mechanism by which CD20 is regulated by the IGF-I receptor. The present results also provide some insight into the nature of the IGF-operated calcium-permeable channel. Despite of the fact that CD20 channel is ectopically expressed in Balb/c 3T3 fibroblasts, the channel is activated by the IGF-I receptor. This raises an interesting possibility that the putative IGF-operated channel and CD20 may share some structural homology.

CD20 is a cell-surface protein expressed in B lymphocytes. Although it has several functions in the signal transduction system in B cells (8–10), information regarding the ligand that activates CD20 is not available. The present results may provide some insight into the regulation of CD20 functions. An obvious candidate ligand that may activate CD20 is interleukin 4, since this cytokine and insulin share the signaling molecules, insulin receptor substrate-1 and -2 (31, 32). However, interleukin 4 does not activate CD20 channels in Raji cells. It is possible that a ligand that acts on a receptor system functionally resembling the IGF-I receptor activates the channel activity of CD20. Alternately, the ligand activating PTX-sensitive G proteins may activate the CD20 channel.

Acknowledgments—We thank Dr. M. Kato of the Nihon Medical College for suggestions and Kiyomi Ohgi for secretarial assistance.

2 M. Kanzaki and I. Kojima, unpublished observations.
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