When the calcium-permeable cation channel CD20 is expressed in Balb/c 3T3 cells, it is activated by insulin-like growth factor-I (IGF-I) via the IGF-I receptor (Kanzaki, M., Nie, L., Shibata, H., and Kojima, I. (1997) J. Biol. Chem. 272, 4964–4969). The present study was conducted to investigate the role of G proteins in the regulation of the CD20 channel. In the excised patch clamp mode, activation of the CD20 channel by IGF-I required GTP, Mg2+, and ATP in the bath solution, and removal of either GTP or ATP attenuated the activation. Non-hydrolyzable ATP could substitute for ATP, and guanyl-5′-yl thiophosphate blocked the activation of the channel by IGF-I. The CD20 channel was also activated by guanosine 5′-O-(thio)triphosphate, and ATP was not required for the activation. Addition of a preparation of Gi2 holoprotein purified from bovine brain activated the CD20, and the β-adrenergic receptor kinase peptide did not affect the number of channel openings induced by the G protein. The CD20 channel was stimulated by the GTP-bound form of recombinant Gα subunit purified from SF9 cells. The Gα subunit was less effective, and the Gβ subunit had no effect. Purified recombinant βγ subunits did not affect the activity of the channel. Finally, IGF-I-induced activation of CD20 was inhibited by an antibody against Gi2α subunit. These findings indicate that the CD20 channel expressed in Balb/c 3T3 cells is activated by the IGF-I receptor via the α subunits of heterotrimeric G proteins.

CD20 is a transmembrane protein with molecular mass of 35 kDa that is expressed in B lymphocytes (1). This protein has been considered to be involved in growth regulation since monoclonal antibodies against CD20 modify the growth rate of the cells (2–4). Molecular cloning of the CD20 protein has provided information on the putative structure, which resembles that of ion channels and transporters (5, 6). Bubien et al. (7) showed that the CD20 channel expressed in fibroblasts functions as a calcium-permeable cation channel. Using whole cell recordings, they showed that CD20 is a voltage-independent cation channel that permeates calcium. Since calcium entry is a prerequisite for the progression through G1 phase (8), we expressed the CD20 channel in Balb/c 3T3 cells and studied the changes in the growth characteristics of the cells (9). Indeed, expression of the CD20 channel resulted in three major alterations in G1 progression induced by insulin-like growth factor-I (IGF-I).1 First, expression of the CD20 channel shortened the period required for the entrance to the S phase by accelerating the G1 progression induced by IGF-I. Second, expression of the CD20 channel reduced the dependence of the G1 progression on extracellular calcium, and CD20-expressing cells could progress toward S phase in medium containing lower concentrations of calcium. Third, expression of the CD20 channel enabled IGF-I alone to induce progression in cells arrested in the G0 phase. In Balb/c 3T3 cells, IGF-I is not capable of stimulating DNA synthesis in G0-arrested cells (10). IGF-I promotes G1 progression only when G0-arrested cells are treated sequentially with platelet-derived growth factor and epidermal growth factor (EGF) (10–12). Therefore, expression of the CD20 channel at least partly reproduces the effect of platelet-derived growth factor and EGF. These results indicate that the expression of CD20 modulates the action of IGF-I. Our recent study revealed that the calcium-permeable channel activity of CD20 is activated by IGF-I in Balb/c 3T3 cells expressing CD20 (13). When IGF-I is added to quiescent cells expressing CD20, the opening probability of the CD20 is markedly augmented, and calcium entry via the CD20 channel is greatly increased. Interestingly, the effect of IGF-I on the CD20 channel is blocked by pertussis toxin (PTX) (13). Conversely, mastoparan, an activator of Gαs (14), activated the CD20 channel, and transfection of gip2, a gene encoding constitutive active form of the α subunit of Gαs (15), also activated the CD20 channel (13). Therefore, it is possible that IGF-I activates the CD20 channel by a mechanism involving a PTX-sensitive G protein. The present study was conducted to address this issue, and the data suggest that the CD20 channel is activated principally by the α subunit of G12.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human IGF-I was supplied by Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan). [32P]dCTP and [125I]labeled protein A were obtained from DuPont NEN. Na[125I] was purchased from Cymbus Pharmaceuticals, Inc. (Irvine, CA). Anti-CD20 antibodies were obtained from Cymbus Bioscience Ltd. (CBL 456; Southampton, UK) and Coulter Immunology (Coulter clone B1; Hialeah, FL). The anti-Gαs α subunit antibody was obtained from Calbiochem (anti-Gi2α) and G12α subunits C-terminal-(345–354) rabbit IgG. Mastoparan was purchased from Peptide Institute, Inc. (Osaka, Japan). Purified Gαs, Gαi1, and Gα12 subunits were used as sources of Gα subunits.

1 The abbreviations used are: IGF-I, insulin-like growth factor-I; G proteins, GTP-binding proteins; EGF, epidermal growth factor; PTX, pertussis toxin; PBS, phosphate-buffered saline; βARK, β-adrenergic receptor kinase; nP50, mean opening probability; GTPγS, guanosine 5′-O-(thio)triphosphate; GDPβS, guanyl-5′-yl thiophosphate; ATPγS, adenosine 5′-O-(thio)triphosphate; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.
was prepared as described previously (16, 17). The α subunits of the G proteins G<sub>α1</sub>, G<sub>α2</sub>, and G<sub>α3</sub> were expressed using the baculovirus/Sf9 insect cell system and purified to homogeneity using DEAE, hydroxyapatite, and Mono P chromatography as described previously (18, 19). The recombinant β<sub>γ</sub> dimers were also expressed using the baculovirus Sf9 insect cell system and purified as described previously (20). The T<sub>a</sub>-peptide of the β-adrenergic receptor kinase (sARK) (21) was synthesized by using peptide synthesizer (Applied Biosystems, Foster City, CA). The crude peptide was purified by preparative high performance liquid chromatography to better than 98% homogeneity as judged by analytical high performance liquid chromatography. All the chemicals were of reagent grade and obtained from commercial sources.

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

**Transfection of cDNA**—The inducible CD20 expression vector (CD20-pMEP4) was transfected into Balb/c 3T3 cells by electroporation as described previously (9). CD20 expressing quiescent Balb/c 3T3 cells were obtained by incubating confluent cells in Dulbecco’s modified Eagle’s medium containing 0.5% platelet-poor plasma and 40 µM ZnCl₂ for 24 h. The constitutively active G<sub>α2</sub> mutant (Gip2) expression vector Gip2-pcDNA I was generously provided by Dr. H. Bourne of UCSF. Balb/c 3T3 cells were co-transfected with CD20-pMEP4 and Gip2-pcDNA I using a transfection reagent N-[1-(2,3-dioxoaloyl)propyl]-N,N,N-trimethylammonium methysulfate (DOTAP, Boehringer Mannheim GmbH, Germany). Hygromycin (Wako Pure Chemicals, Osaka, Japan)-resistant colonies were independently picked up and screened for Northern blotting for high expression of Gip2 and CD20 as described previously (13).

We performed a binding study using a mAb against CD20 and CD20 immunostaining with anti-CD20 antibody to show that the CD20-transfected cells express CD20. For the binding assay, the IgG was iodinated by the chloramine-T method (9) to a specific activity of 0.01 to 0.4 mCi/mg. The antibody against the subunits of the G<sub>α</sub> protein was blotted to an Immobilon membrane (Millipore, Yonezawa, Japan). The antibody against the α subunit of the G<sub>α1</sub> protein was blotted to an Immobilon membrane (Millipore, Yonezawa, Japan). The antibody against the α<sub>2</sub> subunit was used as the primary antibody, and detection was achieved by 125I-labeled protein A (0.2 µCi/ml). The blots were subjected to autoradiography, analyzed using a FUJIX BAS2000, and photographed by a FUJIX Pictography 3000 (Fuji Photo Film, Japan).

Immunostaining was performed as described above except PBS containing 0.1% saponin was used to permeabilize the plasma membrane, and tetramethylrhodamine isothiocyanine-conjugated goat anti-rabbit IgG antibody was used as the secondary antibody.

**Electrophysiological Recordings**—The cell-attached configuration, the micropipettes were filled with a solution containing 110 mM BaCl₂ or CaCl₂, 200 nM tetrodotoxin (Seikagaku-Kogyo, Japan), and 10 mM HEPES (pH 7.4, adjusted by adding NaOH) or Ca(OH)₂. In some experiments, Ca<sub>2+</sub> was replaced with 10 mM Ba<sup>2+</sup>. The channel solution contained 137 mM NaCl, 5 mM KCl, 1.1 mM MgCl₂, 1.25 mM CaCl₂, 5 mM glucose, and 10 mM HEPES (pH 7.4, adjusted with NaOH). Single channel recordings were analyzed by using the “EP ANALYSIS” (HEKA) and Igor Pro (Wave Metrics, Lake Oswego, OR). 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, NP<sub>0</sub> (number of channels multiplied by the open probability) was calculated as shown in Equation 1.

\[
NP_0 = \sum_{n=0}^{N} \frac{N!}{n!(N-n)!} t_n \quad (\text{Eq. 1})
\]

where T is the total record time, n is the number of channels open, and t<sub>n</sub> is the recording time during which n channels are open. Therefore, NP<sub>0</sub> 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. All currents were measured with holding potential at ~80 mV unless otherwise mentioned. In the experiments using purified or recombinant G proteins, detergent (CHAPS) concentrations ranged from 0.01 to 0.4 µM (6.14–24.59 ng/ml). To activate purified G protein holotrimers or recombinant α subunits, the proteins were incubated with an equimolar amount of GTP-S for 15 min at 25 °C and applied to the bath solution. This concentration of GTP-S alone was used as control and had no effect (see legend to Figs. 5 and 6).

**Statistical Analysis**—The data are expressed as means ± S.E., and differences between them were analyzed using Student’s t test and analysis of variance. Results were considered to be significantly different when p < 0.05.

**RESULTS**

**Effect of GTP and ATP on IGF-I-induced CD20 Activation in Inside-out Patches**—Previous experiments have shown that the activity of the CD20 channel in a cell-attached patch is observed when IGF-I is added to the pipette solution and that IGF-I-induced CD20 channel activation is abolished by pretreatment with PTX (13). These findings suggest that IGF-I activates CD20 via a PTX-sensitive G protein. To investigate whether or not IGF-I-induced CD20 channel activation is mediated by a G protein, we performed experiments using inside-out patches. Fig. 1A shows the results of a representative experiment obtained in an excised patch. The pipette solution contained 110 mM BaCl₂ and 1 mM IGF-I. After the patch excision, the single channel currents disappeared even in the presence of 0.4 mM GTP and 4 mM Mg<sup>2+</sup> in the bath solution. However, the channel activity appeared again by the subsequent application of 1 mM ATP to the bath. To quantitate the effects of GTP and ATP, we calculated the mean open probability. Fig. 1B shows the mean open probability of the CD20 channel. As depicted, the CD20 channel openings were rarely detected in the bath solution containing GTP and Mg<sup>2+</sup>, but the channel openings were observed when ATP was added to the bath solution. Similarly, channel activity was not observed in the excised patch when only ATP and Mg<sup>2+</sup> were in the bath, and activity was restored when GTP was added to the bath (Fig. 1C). Fig. 1D shows the open probability of the CD20 channel. Similar Ba<sup>2+</sup>-permeable channels resistant to the inhibitory solution containing ATP, GTP, and Mg<sup>2+</sup> were observed in 17/31 inside-out experiments. In experiments without IGF-I in the pipette solution, the current was not observed even if the bath solution contained GTP, Mg<sup>2+</sup>, and ATP. We assumed that these channels were CD20 since the channel events in either cell-attached or inside-out patches were blocked when a monoclonal antibody against CD20 (CBL456; 2 µg/ml) was present in the pipette solution (see Table I). Table I shows the

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The number of cells in which CD20 was activated by each treatment. In the presence of ATP, GTP, and Mg\(^{2+}\) in the internal side of the membrane, CD20 channel was activated in about 50% of the cells. The open probability also decreased in inside-out patches compared with cell-attached patches. NP\(_o\) values were 0.44 \(\pm\) 0.11 and 0.21 \(\pm\) 0.07 in cell-attached and inside-out patches, respectively. The single channel conductance was about 7 pS in excised patches, and this size was similar to those in cell-attached patches (13). None of these channel events in inside-out patches were observed in the absence of Mg\(^{2+}\) in the bath solution. Channel activation was not detected by adding GDP instead of GTP (data not shown). Furthermore, pretreatment of the cells with PTX (100 ng/ml for 4 h) abolished the channel activation both in cell-attached and in inside-out patches.

Effect of the GTP Analogue, GTP\(_{\gamma}\)S, on CD20 Activation in Inside-out Patches—Next, we examined the effect of a non-hydrolyzable GTP analogue, GTP\(_{\gamma}\)S, on CD20 activation in inside-out patches. The pipette solution did not contain IGF-I. Fig. 2 shows the effect of GTP\(_{\gamma}\)S added to the internal side of the plasma membrane on CD20 channel activity. The channel events were not observed in the cell-attached patch because of the absence of IGF-I (Fig. 2, top trace). After the patch excision, the activity of the channel was markedly stimulated by adding 0.1 mM GTP\(_{\gamma}\)S alone but not by GDP\(_{\gamma}\)S (data not shown) in the presence of 4 mM Mg\(^{2+}\). The effect of GTP\(_{\gamma}\)S was dose-dependent and, at concentrations less than 1 \(\mu\)M, GTP\(_{\gamma}\)S did not activate the CD20 channel (data not shown). The channels activated by GTP\(_{\gamma}\)S had identical conductance to the CD20 channel stimulated by IGF-I (pipette), GTP, and ATP (bath) in inside-out patches. Channel events induced by GTP\(_{\gamma}\)S were

![Image](http://www.jbc.org/)

**FIG. 1.** Effect of nucleotides on IGF-I-induced CD20 activation in inside-out patches. Single channel Ba\(^{2+}\) currents were recorded by cell-attached and inside-out patches with a holding potential of \(-80\) mV. A, the channel events of the CD20 protein detected in the cell-attached patch with 1 nM IGF-I in the pipette. The same patch was excised (inside-out) and applied to the bath solution containing 0.4 mM GTP and Mg\(^{2+}\). Then 1 mM ATP was subsequently added to the bath solution. B, NP\(_o\) calculated from the experiments described in A. Values are the means \(\pm\) S.E. for 16 patches obtained in three different experiments. C, the channel events of the CD20 protein detected in the cell-attached patch with 1 nM IGF-I in the pipette. The same patch was excised (inside-out) and incubated in the bath solution containing ATP and Mg\(^{2+}\). Then, GTP was subsequently added to the bath solution. D, NP\(_o\) calculated from the experiments described in C. Values are the means \(\pm\) S.E. for 15 patches in three different experiments.

**TABLE I**

CD20 channel activation in cell-attached and inside-out patches

|                  | Cell-attached patches | Inside-out patches |
|------------------|-----------------------|--------------------|
|                  | ATP + GTP - Mg\(^{2+}\) | GTP + Mg\(^{2+}\) | Mg\(^{2+}\) |
| None             | 0/29                  | 0/26               | 0/26       |
| IGF-I            | 31/34                 | 17/31              | 4/31       | 1/31       |
| IGF-I mAb        | 1/19                  | 1/16               | ND         | ND         |
| IGF-I (PTX-treated) | 0/21               | 1/17               | ND         | ND         |

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abandoned by the mAb against CD20 (data not shown). ATP in the internal side of the membrane was required for IGF-I-induced CD20 activation. As shown in Table II, however, ATP was not required and had no stimulatory effect on GTPγS-activated CD20 channel events.

**Effect of ATP on Mastoparan-induced and Gip2-induced CD20 Activation in Inside-out Patches**—As shown in the above sections, ATP on the internal side of the plasma membrane was required for IGF-I-induced but not for GTPγS-induced CD20 activation in inside-out patches. These results suggest that ATP is essential for IGF-I-induced CD20 activation but is not necessary for CD20 activation when G protein is activated directly. To evaluate this possibility, we examined whether or not ATP is necessary for CD20 activation induced by mastoparan. As shown in Fig. 3A, GTP and Mg2+ on the internal side of the membrane were required and sufficient for CD20 channel activation with mastoparan in the pipette (21/22 patches). Likewise, when Gip2, a constitutively active mutant of Gαi2, was co-expressed with CD20, ATP was not necessary to activate CD20 (19/19 patches). These channel openings were not augmented by ATP in each condition (Table II). On the other hand, Mg2+ was necessary for CD20 activation, and these channel events disappeared when Mg2+ was removed by adding EDTA (data not shown). Furthermore, in cells activated by mastoparan, CD20 channel events disappeared when GTPγS was added to the internal side of the membrane (Fig. 3B). Similarly, the CD20 channel activity in Gip2-transfected cells was blocked by EDTA (Fig. 3C).

**Effect of ATP Analogues on CD20 Activation in Inside-out Patches**—As indicated above, ATP was necessary for IGF-I to activate the CD20 channel in addition to GTP and Mg2+ in excised patch. Since the IGF-I receptor has intrinsic tyrosine kinase activity in the intracellular domain of the β subunit, it is an interesting question whether or not protein phosphorylation is involved in the IGF-I-induced CD20 activation. To address this question, we examined the effect of non-hydrolyzable analogues of ATP, ATPγS and AMP-PNP, on IGF-I-induced CD20 activation in inside-out patches. The pipette solution contained 1 nM IGF-I. As shown in Fig. 4A, the channel activity was detected upon application of either 1 mM AMP-PNP or 1 mM ATPγS in the presence of 0.4 mM GTP and 4 mM Mg2+. NPγS activated by either ATPγS or AMP-PNP was slightly higher than that by ATP (Fig. 4B). In the absence of GTP and Mg2+, neither ATPγS nor AMP-PNP alone had a stimulatory effect. These results suggest that ATP binding rather than hydrolysis of ATP is necessary for IGF-I-induced activation of the G protein.

**Effect of Purified G Protein on CD20 Activation**—The present findings as well as those of our previous study (13) indicate that the CD20 channel is regulated by G protein(s). To further assess the involvement of G protein in the regulation of the CD20 channel, we investigated the effect of reconstituting purified G proteins obtained from bovine brain into the excised patches. The preparation used in these experiments contains heterotrimeric Gαi and Gβγ proteins. The pipette solution did not contain IGF-I. The purified G protein was pretreated with an equimolar concentration of GTPγS. As shown in Fig. 5, the buffer solution without G protein did not activate the channel, but the GTPγS-activated brain G protein (20 nM) applied to the internal side of CD20-expressing cell membranes induced persistent openings of the Ca2+-permeable channel. The channels activated by purified G protein had identical conductance as the CD20 channel stimulated by IGF-I (pipette), GTP and ATP (bath), or GTPγS (bath) alone. These single channel currents were abolished by the mAb against CD20 (data not shown). The G protein-induced activation of the CD20 channel was observed in all patches tested (16/16 patches in three different experiments). Neither GTPγS alone added at 20 nM nor the GDF-bound form of the G proteins activated the channel.

**Effect of βARK Peptide on CD20 Activation in Inside-out Patches**—The observation that the expression of Gip2 results in CD20 channel activation (13) suggests the importance of the α subunit of the G protein to activate the CD20 channel. Since both α-GTP and βγ can activate multiple effectors (23), we examined the effect of the C-terminal peptide of the βARK peptide to assess the possibility that the βγ subunit might be activating the CD20 channel in the excised patches. The C-terminal domain of βARK would be expected to interact with the βγ subunit of the activated G protein and neutralize its action (21). As shown in Fig. 5, the G protein-activated CD20 channel event was not affected by 10 μM βARK peptide (a 500-fold excess of peptide). The αARK peptide had no significant effects on channel events in various conditions using IGF-I, mastoparan, or GTPγS to activate the channel or using patches activated by co-expression of Gip2 (data not shown). These results suggest that the α subunits and not the βγ dimers have a stimulatory effect on the CD20 channel.

**Effect of Recombinant G Protein Subunits on CD20 Activation**—To confirm the involvement of the α subunit of Gαi in CD20 activation and to clarify the subtype of αi subunit that activates the CD20 channel, we investigated the stimulatory action of recombinant α subunits of G11, G12, and G13 on CD20...
channel activity in inside-out patches. As shown in Fig. 6, the CD20 channel was activated by reconstitution of 1 nM recombinant \( \alpha_{i2} \) and \( \alpha_{i3} \) (GTP\( \gamma \)S-activated) but not by \( \alpha_{i1} \). Activation of the CD20 channel by recombinant \( \alpha_{i} \) subunits was normalized to the activity induced by 5 nM \( \alpha_{i2} \) subunit. As shown in Fig. 6B, \( \alpha_{i2} \) was the most potent. The minimal concentration of \( \alpha_{i2} \) required to activate the channel was approximately 250 pM, and the EC\(_{50}\) was about 6 nM. The \( \alpha_{i3} \) subunit was less effective than the \( \alpha_{i2} \) subunit and \( \alpha_{i1} \) had essentially no effect. As expected from the data shown in Fig. 5, 20 nM recombinant \( \beta_{1,\gamma2} \) which could activate PLC\( \beta_2 \) (24), had no significant effect on CD20 channel activity (Fig. 6C). These channel events activated by recombinant \( \alpha_{i2} \) subunits were not affected by recombinant \( \beta_{1,\gamma2} \). Neither GTP\( \gamma \)S alone (concentrations up to 50 nM) nor the detergent solution used as a vehicle for the recombinant proteins affected the channel activity. Exposing the cytoplasmic side of the membrane patches to exogenously applied ATP did not modify the channel events (data not shown).

**FIG. 4. Effect of ATP analogues on IGF-I-induced CD20 activation in inside-out patches.** A, single channel current was measured in inside-out patches with 1 nM IGF-1 in the pipette. The bath solution contained 0.4 mM GTP, 4 mM Mg\(^{2+}\), and ATP analogues as indicated. B, \( nP_\text{o} \) was calculated from the experiments described in A. \( nP_\text{o} \) was normalized to that obtained with 1 mM ATP. Values are means ± S.E. for 10 patches in three different experiments.

**FIG. 5. CD20 channel activation by purified brain G protein.** Single channel current was measured in inside-out patches. Purified G protein was preactivated with the equimolar concentration of GTP\( \gamma \)S (20 nM). G protein and \( \beta\)ARK peptide (10 \( \mu \)M) were applied as indicated.
The CD20 protein functions as a calcium-permeable cation channel (7, 9) and, when expressed in Balb/c 3T3 cells, alters the growth characteristics of the cells (9). In particular, expression of the CD20 channel modifies the ability of IGF-I to stimulate progression (9). This alteration is due at least in part to the activation of the CD20 channel by IGF-I via the IGF-I receptor (13). Interestingly, although the IGF-I receptor is very similar to the insulin receptor (25), the IGF-I-mediated activation of CD20 is blocked by PTX. The present study was conducted to examine which G proteins might be involved in the IGF-I-mediated activation of the CD20 channel. We studied the activity of CD20 in excised patches in which the condition of the cytoplasmic face of the plasma membrane could be manipulated by changing the bath solution. Activation of the channel by IGF-I in the cell-attached mode was examined first, and the existence of the ligand, receptor, channel, and transducers in the patch was confirmed. The following observations combined with our previous results (13) indicate that IGF-I activates the CD20 channel by a G protein-dependent mechanism. First, IGF-I-induced activation of the CD20 required GTP as well as Mg$^{2+}$ and removal of either GTP or Mg$^{2+}$ attenuated the activation. Second, GDP$\beta$S, which inactivates G proteins, inhibited the IGF-I action on CD20. Third, G protein-mediated activation of the CD20 channel was directly demonstrated by adding purified G protein (G$_i$/G$_o$ class) purified from the brain. Like the calcium-permeable cation channel activated by erythropoietin (26), calcium-permeable voltage-independent cation channel CD20 is regulated by G$_i$ proteins.

There are abundant data to show that ion channels are regulated by activation of G protein-coupled receptors. The signaling pathways used include second messengers, phosphorylation, and direct regulation by G proteins (27, 28). Currently, K$^+$, Ca$^{2+}$, and Cl$^-$ channels are thought to be regulated by either the $\alpha$ or $\beta\gamma$ subunits of G proteins (27, 29). For example, it is now clear that the muscarinic K$^+$ channel in the atrium is regulated by G protein $\beta\gamma$ subunits (27, 30) and there is growing evidence that the N-type Ca$^{2+}$ channel is also regulated by these subunits (27, 28, 31, 32). In contrast, the Cl$^-$ channels in renal epithelial cells may be regulated by a G$_i$ $\alpha$ subunit (27). The data in this report clearly suggest that the CD20 channel is also regulated by members of the G$_i$ $\alpha$ family of proteins. Interestingly, only the G$_{i2}$ $\alpha$ subunit is fully effective in activating this channel, whereas G$_{i1}$ protein is essentially inactive. The particular preparations of G$_{11}$, G$_{i2}$, and G$_{i3}$ $\alpha$ subunits used in this study are fully active as judged by their efficacy in other assays. For example, all three G$_{i}\alpha$s couple equally well to recombinant A$_2$ adenosine receptors (33). When a blocking antibody against G$_{i2}$ and G$_{i3}$ was added, the IGF-I-induced activity was markedly inhibited. Therefore, the CD20 channel is principally regulated by G$_{i2}$ in Balb/c 3T3 cells. Considered together, these results suggest that the interaction between the CD20 Ca$^{2+}$-permeable channel and the different G$_i$ $\alpha$ subunits is very selective.

While the multiple variations of the mitogen-activated protein kinase pathway are thought to be the major signaling mechanisms used by receptors with tyrosine kinase activity (35, 36), the finding that receptors in this family can also couple to G protein $\alpha$ subunits is not unique. A number of investigators have shown that responses to both insulin and EGF receptors may be elicited via G protein-coupled mechanisms. In rat hepatocytes, the ability of EGF to stimulate inositol lipid

**FIG. 6.** CD20 channel activation by recombinant $\alpha$ subunits of G$_i$. A, single channel current was measured in inside-out patches. Recombinant G$_i\alpha$ subunits were preactivated with an equimolar concentration of GTP$\gamma$S. $\alpha$ subunits (1 nm) were applied sequentially as indicated. B, NP$_a$ calculated from patches treated with various concentrations of $\alpha$ subunits was calculated. NP$_a$ was normalized to the activity obtained by 5 nm $\alpha_{i2}$. Values are the means $\pm$ S.E. for more than nine patches in each condition. C, single channel current was measured in inside-out patches. Recombinant $\beta_{i2}$ and $\alpha_{i2}$ were applied as indicated.

**DISCUSSION**

The CD20 protein functions as a calcium-permeable cation channel (7, 9) and, when expressed in Balb/c 3T3 cells, alters the growth characteristics of the cells (9). In particular, expression of the CD20 channel modifies the ability of IGF-I to stimulate progression (9). This alteration is due at least in part to the activation of the CD20 channel by IGF-I via the IGF-I receptor (13). Interestingly, although the IGF-I receptor is very similar to the insulin receptor (25), the IGF-I-mediated activation of CD20 is blocked by PTX. The present study was conducted to examine which G proteins might be involved in the
breakdown in intact cells or to stimulate the binding of GTP$\gamma$S to isolated membrane preparations is blocked by pertussis toxin, suggesting that the receptor couples to the $\alpha_i\beta$ subunit in these cells (37, 38). Moreover, in rat cardiomyocytes, EGF stimulates adenyl cyclase by coupling to the $\alpha_i$ protein (39). In BC3H-1 myocytes, the ability of insulin to generate diacylglycerol can be blocked by pertussis toxin (40).

The IGF-I receptor resembles the insulin receptor and has intrinsic tyrosine kinase activity in its $\beta$ subunit (25). Binding of the ligand to the $\alpha$ subunit leads to the activation of the receptor kinase, which phosphorylates its own subunit, and autophosphorylation of the receptor up-regulates the activity of receptor kinase, thus phosphorylating other substrates including insulin receptor substrate-1 (see Ref. 41 for review). An interesting question is whether or not receptor kinase activity is necessary for the channel activation. The present results show that non-hydrolyzable analogues of ATP reproduced the effect of ATP. Thus, ATP binding rather than hydrolysis may be necessary for the receptor-mediated activation of the G protein. It should be mentioned that, in our experiments, the cell was first activated by IGF-I added inside the patch in the cell-attached mode. It is likely that autophosphorylation of the receptor took place in the beginning of the experiment. However, it is not certain whether or not the receptor remained autophosphorylated in the excised patch. In any event, based on the results obtained with AMP-PNP, ATP binding rather than hydrolysis is necessary for the channel activation. In this regard, we recently found that insulin activates calcium-permeable cation channel in Chinese hamster ovary cells expressing human insulin receptor. Interestingly, insulin-induced activation of the channel requires GTP, ATP, and Mg$^{2+}$ in the excised mode of the patch clamp, and non-hydrolyzable ATP analogues can be substituted for ATP. In the case of the insulin receptor, ATP binding is known to induce alterations of the conformation of the active domain of the receptor (42). This raises the possibility that receptor-mediated activation of the $\alpha$ subunit induces conformational changes in the $\beta$ subunit of the receptor, which is necessary for the activation of the $\alpha_i\beta$ subunit. This possibility should be examined experimentally in the future.

CD20 is expressed in B lymphocytes (1, 2). The present results provide insights into the regulation of CD20 in lymphocytes and regulation of the IGF-operated channel in Balb/c 3T3 cells. Since the CD20 channel can be activated by $\alpha_{i2}$, other receptors coupled to the $\alpha_i\beta$ heterotrimer should be expected to activate the channel in B lymphocytes. Besides the function as a calcium-permeable channel, CD20 also functions in the phosphorylation cascade (43). It is an interesting question whether or not the latter function is also modulated by the $\alpha_i\beta$ protein. In Balb/c 3T3 cells, IGF-I stimulates calcium entry by activating the IGF-operated calcium-permeable channel (44), and regulation of the IGF-operated channel resembles in many respects that of CD20. The IGF-induced calcium entry is blocked by PTX (45) and is augmented by GTP$\gamma$S (46). Since IGF-I activates $\alpha_i$ (34), it seems likely that the regulatory mechanism of the IGF-operated channel is similar to that of CD20 expressed in Balb/c 3T3 cells.

In summary, the CD20 channel expressed in Balb/c 3T3 cells is activated by IGF-I. Activation of the CD20 channel is principally caused by the $\alpha$ subunit of $\alpha_{i2}$.

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