Cloning and characterization of a bifunctional metabotropic receptor activated by both extracellular calcium and glutamate

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Received 24 June 1996

Abstract A cDNA from salmon brain which induces a consistent activation of Ca^{2+} dependent Cl^- (Ca^{2+}-Cl^-) current in *Xenopus* oocytes in the saline solution was isolated by an expression cloning method. The primary structure showed a high homology with the metabotropic glutamate receptors (mGluR), and a limited homology with the Ca^{2+}/polyvalent cation receptors (CaR). The encoded protein was functionally characterized for the sensitivity to extracellular Ca^{2+} (Ca^{2+}_o), and the consistent activation of Ca^{2+}-Cl^- current was determined to be due to Ca^{2+} contained in the saline solution. It was also confirmed that the encoded protein has a function to sense glutamate. An increase in inositol 1,4,5-triphosphate turnover was observed when either Ca^{2+} or glutamate was applied. Thus, the encoded protein was identified as a bifunctional metabotropic receptor for both Ca^{2+} and glutamate. In addition, rat mGluR1α was also shown to be activated by Ca^{2+}_o. This is the first report of a G-protein coupled receptor which is activated by two totally different physiological agonists.

Key words: Bifunctional; Metabotropic; Receptor; Calcium; Glutamate; cDNA; Cloning

1. Introduction

A wide variety of GTP binding protein (G-protein) coupled receptors has already been isolated. However, a G-protein coupled receptor which is activated by multiple totally different physiological agonists is not known [1]. In this paper, we report the isolation and functional characterization of a cDNA from salmon brain which encodes a bifunctional metabotropic receptor for both extracellular Ca^{2+} ($Ca^{2+}{}_o$) and glutamate (sBimR: salmon bifunctional metabotropic receptor).

Xenopus oocytes possess Ca^{2+} dependent Cl^- (Ca^{2+}-Cl^-) channels [2], and have been used as a convenient system to monitor the increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) caused by an activation of G-protein coupled receptors [3]. Using this system for screening, the first cDNA clone of the metabotropic glutamate receptor (mGluR1α) was isolated [4] and various members of the same family were cloned by the sequence homology (mGluR2–8) [5–9]. The Ca^{2+} sensing receptor (CaR) of the parathyroid was also isolated by expression cloning and found to belong to the G-protein coupled receptor family [10]. By the sequence homology, members of the CaR family were isolated from the kidney [11] and also from the brain [12]. The structural similarity of CaR and mGluR, especially mGluR1, has already been noticed by some investigators [9–13]. The characteristic feature these two receptor families have in common is the presence of a long N-terminal cytoplasmic chain. However, it is not yet known whether mGluR actually functions as a Ca^{2+} receptor or not.

We constructed a cDNA library from salmon brain with a view to isolating a G-protein coupled hormonal receptor by expression cloning [14]. In the course of the electrophysiological screening, we happened to notice that a pool of the cDNA library induced an increase in the basal level of the Ca^{2+}-Cl^- current without applying any agonist, and isolated the causative cDNA clone (cDNA for sBimR). The basal activity was found to be due to the activation of sBimR by Ca^{2+} contained in the bath solution. The primary structure exhibited a high degree of homology with mGluR1α [4], and was characterized to be activated by glutamate.

2. Materials and methods

2.1. Molecular biology

Poly $(A)^+$ RNA was isolated from the brain of the male breeding season masu salmon (*Oncorhynchus masou*) using a Fast Track RNA isolation kit (Invitrogen), and fractionated as described previously [15]. A unidirectional cDNA library was constructed as described previously [16]. In vitro transcription of cRNA was done as described previously [15]. DNA sequence was determined using the PRISM sequence reaction kit (Applied Biosystems) and a DNA sequencer (Applied Biosystems, 373A). Alignment of the deduced amino acid sequence was done by the PCGENE program based on the maximum matching algorithm of Higgins and Sharp [17].

Northern hybridization was performed at high stringency as described previously [16]. The entire 5.5 kb sBimR cDNA was labeled with ^{32}P by random priming and used as a probe.

Preparation of frozen sections of the salmon brain and hybridization at high stringency were performed as described previously [16]. Digoxigenin labeled anti-sense and sense strand probes were prepared from the entire 5.5 kb sBimR cDNA clone using the DIG labeling kit (Boehringer Mannheim).

2.2. Electrophysiology

Preparation of *Xenopus* oocytes and two electrode voltage clamp experiments were carried out as described previously [16]. The standard saline solution used in the oocyte culture, in the screening (Fig. 2a) and in the measurement of IP_3 (Fig. 3c) contained 88 mM NaCl, 1 mM KCl, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.8 mM $MgSO_4$, 2.4 mM $NaHNO_3$ and 15 mM HEPES, pH 7.6. In the experiments of applications of $Ca^{2+}{}_o$ or other polyvalent cations (Fig. 2b–d) and glutamate (Fig. 3a,b), Ca^{2+} free saline (0 mM Ca^{2+}, 0.1 mM Mg^{2+}) was used as a bath solution to reduce the basal level of the Ca^{2+}-Cl^- current. 0.1 mM Mg^{2+} was kept to minimize a leak current level. To

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Abbreviations: sBimR, salmon bifunctional metabotropic receptor; mGluR, metabotropic glutamate receptor; CaR, Ca^{2+} sensing receptor; $Ca^{2+}{}_o$, extracellular Ca^{2+}; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; $Ca^{2+}{}_i$, intracellular Ca^{2+}; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; Ca^{2+}-Cl^- channel, Ca^{2+} dependent Cl^- channel; G-protein, GTP binding protein; IP_3, inositol 1,4,5-triphosphate

PII S0014-5793(96)00786-7

monitor the time course of the response, depolarizing pulses of 175 ms to +60 mV from the holding potential of −80 mV were applied repeatedly every 2 s. While giving repeated depolarizing pulses, 1/50 volume of 50 times concentrated stock of Ca^{2+} and other polyvalent cations (Fig. 2b–d) or 1/10 volume of 10 times concentrated stock of glutamate (Fig. 3a,b) was applied gently to the bath so that the flow was not directed at the oocyte, and then mixed immediately and thoroughly by pipetting.

2.3. Measurements of inositol 1,4,5-triphosphate

Oocytes injected with water, sBimR cRNA or the rat mGluR1α cRNA were preincubated for 30 min in the Ca^{2+} free saline solution, transferred into either the standard saline solution (0.7 mM Ca^{2+} and 0.8 mM Mg^{2+}), or the saline solution with high Ca^{2+} (10 mM) or glutamate (100 μM) and incubated at room temperature for 5 min. The solutions were then almost completely removed and the oocytes were immediately frozen by liquid nitrogen. Oocytes in each group were homogenized in 10% trichloroacetic acid [4,18]. IP_3 was measured using an IP_3 [^{3}H] assay system (Amersham) following the instruction attached to the kit.

3. Results

Xenopus oocytes are known to possess Ca^{2+}-Cl^- channels, which are more active at depolarized potentials [2]. The characteristic slow activation of the current with depolarization is used as a good indicator of Ca^{2+}_i [2]. While we were performing an expression cloning of a hormonal receptor of the salmon (*Oncorhynchus masou*) brain, we happened to notice that injection of cRNA of a subpool of the cDNA library induced a consistent activation of Ca^{2+}-Cl^- current without application of an extracellular agonist (Fig. 2a), and the causative single clone (cDNA for sBimR) was isolated.

The isolated cDNA was approximately 5.5 kb in size and contained one open reading frame which encodes 1218 amino acids (Fig. 1). It belongs structurally to the G-protein coupled receptor family, with seven putative transmembrane segments [1]. The deduced amino acid sequence showed a high degree of homology to the mGluRs [4–9], especially to mGluR1α [4] (69% identity of amino acids), and a limited but significant homology to the CaR [10–13] (24% identity of amino acids) (Fig. 1).

The function as a metabotropic glutamate receptor of sBimR could not explain the increased basal level of Ca^{2+}-Cl^- current, because no glutamate was contained in the bath solution. Since sBimR showed structural homology with CaR, and since 0.7 mM Ca^{2+} and 0.8 mM Mg^{2+} were contained in the saline solution in the bath, it was hypothesized that sBimR might be activated by extracellular polyvalent cations, similarly to CaR [10]. When a Ca^{2+} free saline solution was used as a bath solution, the amplitude of the Ca^{2+}-Cl^- current decreased, and application of Ca^{2+} to the bath to give a final concentration of 2 mM evoked Ca^{2+}-Cl^- current after a la-

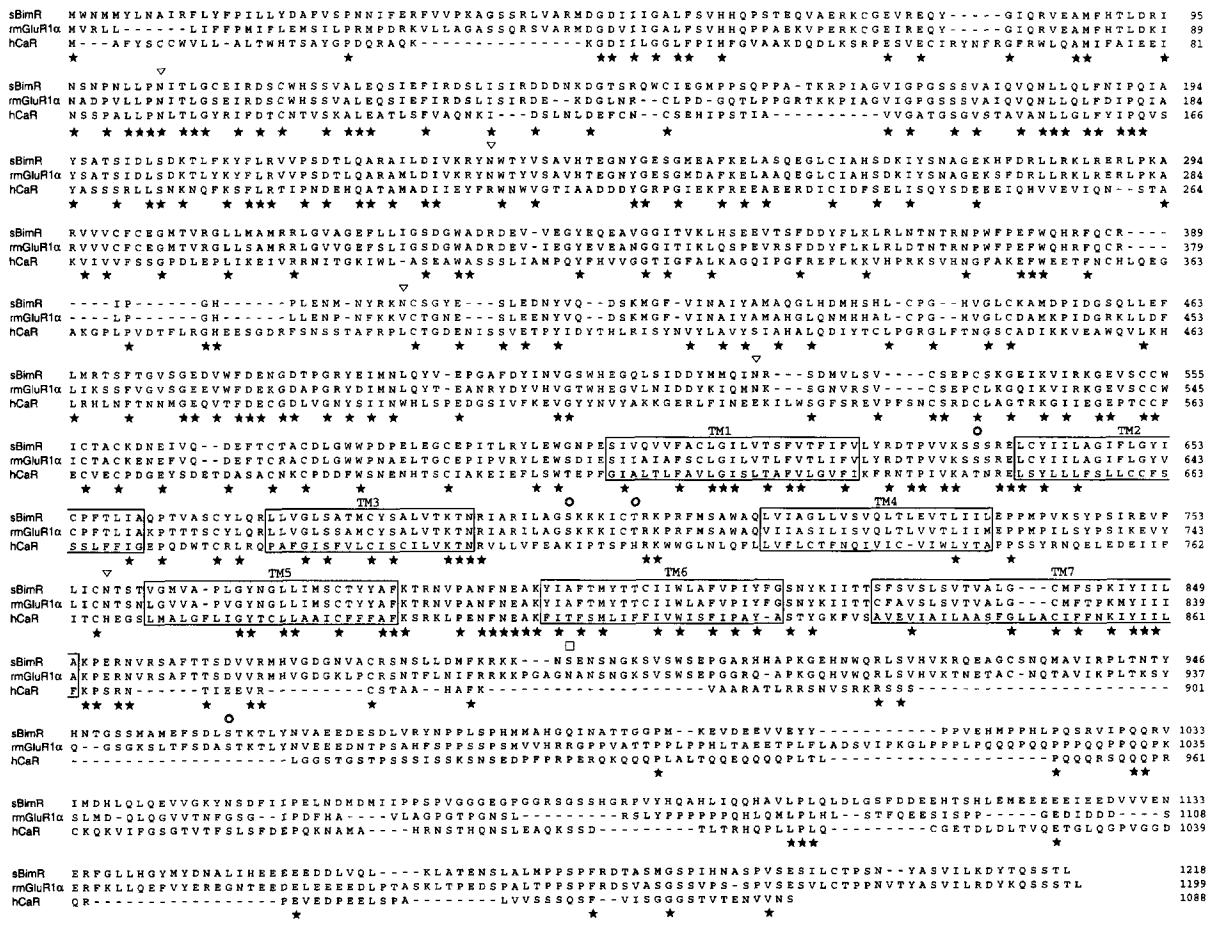

Fig. 1. Alignment of the deduced amino acid sequences of sBimR with the rat mGluR1α [4] and the human CaR [13]. (−) indicates gaps inserted to increase the matching. Amino acids identical in the three cDNAs are indicated below the sequences by stars. Seven putative transmembrane segments (TM1 to TM7) are indicated by boxes. In the sBimR, there was one potential cAMP phosphorylation site (open square: S892), four potential protein kinase C phosphorylation sites (double circles: S636, S699, T705 and S961) and five potential *N*-glycosylation sites (inverted open triangles: N104, N233, N403, N525 and N757), which are marked above the sequence.

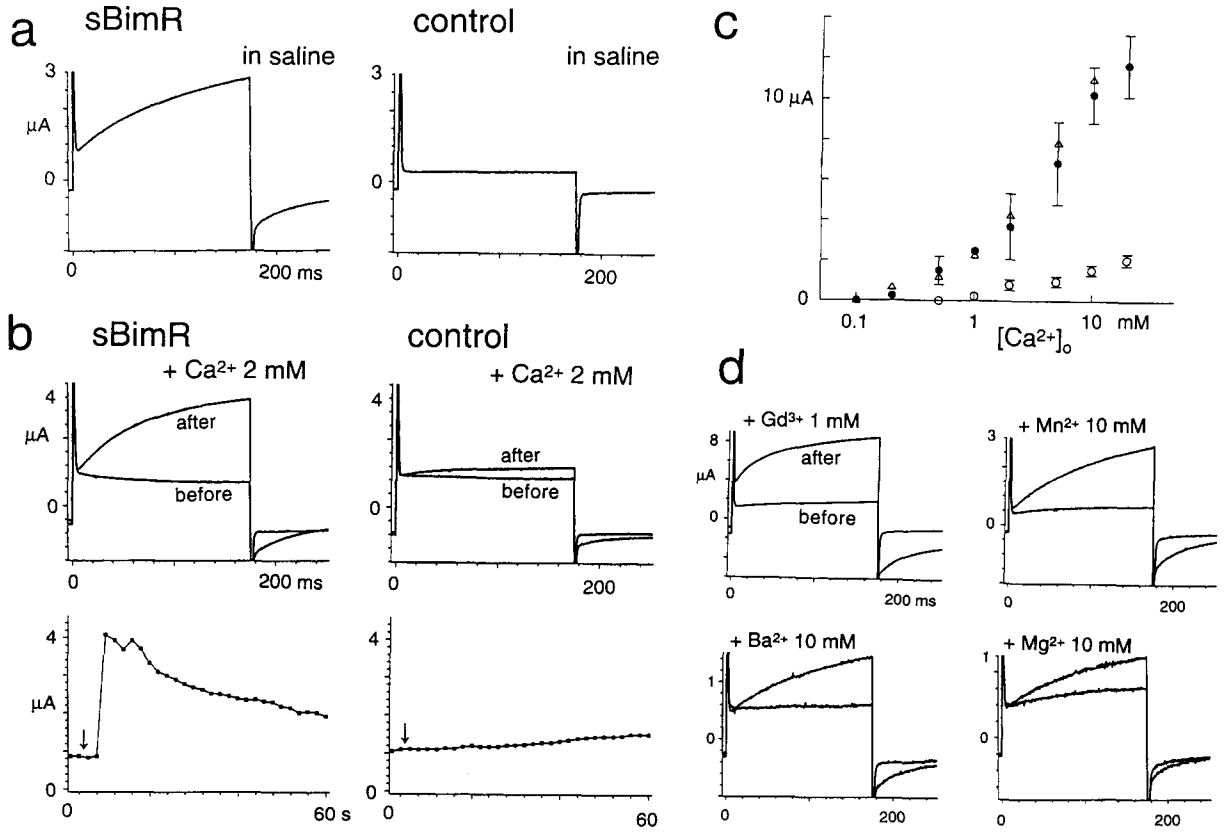

Fig. 2. Activation of Ca^{2+}-Cl^- current by extracellular polyvalent cations in *Xenopus* oocytes injected with sBimR cRNA. (a) Presence of Ca^{2+}-Cl^- current in *Xenopus* oocytes injected with sBimR in the standard saline solution containing 0.7 mM Ca^{2+} and 0.8 mM Mg^{2+} (left). As the Ca^{2+}-Cl^- channel is less active at hyperpolarized potential, the presence of Ca^{2+}-Cl^- current is not clear when the membrane potential is held at −80 mV (see the current level of the short segment of the trace before 0 ms). However, upon applying a depolarizing pulse from −80 mV to +60 mV, a Ca^{2+}-Cl^- current with a characteristic slow activation (the segment in the middle) and deactivation (the segment at the end) kinetics was clearly detected (left). The current was not detected in the control oocytes injected with water (right). (b) Ca^{2+}-Cl^- current of *Xenopus* oocytes injected with sBimR cRNA (b, left) induced by application of Ca^{2+} to the bath. The oocytes were put in the Ca^{2+} free saline solution to decrease the basal level of Ca2+-Cl^- current observed in the standard saline solution. The Ca^{2+}-Cl^- current amplitude was monitored by applying depolarizing pulses every 2 s before, during and after the addition of Ca^{2+}. Two current traces before the application of Ca^{2+}, and of the largest amplitude after the application were shown (upper). The current amplitudes at the end of each depolarizing pulse are plotted to show the time dependence of the response. The time when Ca^{2+} was added is shown by the arrow (lower). Oocytes injected with water also showed a gradual and slight response to application of Ca^{2+} (right). (c) Dose-response curves of the Ca^{2+}-Cl^- current for $[Ca^{2+}]_o$. Data are shown for oocytes injected with sBimR cRNA (filled circles and error bars: mean and standard deviation, $n = 3$–6), water (open circles and error bars: mean and standard deviation, $n = 2$–4) and the rat mGluR1α (open triangles, $n = 1$). (d) Ca^{2+}-Cl^- current of oocytes injected with sBimR cRNA induced by application of Gd^{3+} (1 mM), Mn^{2+} (10 mM), Ba^{2+} (10 mM) or Mg^{2+} (10 mM) to the bath solution. The oocytes were placed in the Ca^{2+} free saline solution, following which the polyvalent cations were added to the bath solution. Two current traces, before the application of polyvalent cations, and at the peak of the induced response after the application, are shown.

tency of a few seconds (Fig. 2b). In the water injected oocytes, increasing $[Ca^{2+}]_o$ also produced a slight increase of the Ca^{2+}-Cl^- current after a long latency, which could be a consequence of gradual influx of Ca^{2+}, for example, through voltage-gated Ca^{2+} channels (Fig. 2b). The dose-response curve of the Ca^{2+}-Cl^- current in the oocytes injected with the sBimR cRNA for $Ca^{2+}{}_o$ showed that the steep part of the curve is in the physiological range of $[Ca^{2+}]_o$ (Fig. 2c, filled circles and error bars). Other polyvalent cations (Gd^{3+}, Mn^{2+}, Ba^{2+} and Mg^{2+}), which are known to be agonists for the CaR [10], also produced Ca^{2+}-Cl^- current in the cRNA injected oocytes (Fig. 2d) but not at all in the water injected oocytes (data not shown). The order of effectiveness of different cations ($Gd^{3+} > Ca^{2+} > Mg^{2+}$) was virtually identical with that of CaR. The effectiveness of polyvalent cations other than Ca^{2+} proved that the activation of Ca^{2+}-Cl^- current is not caused by the direct influx of Ca^{2+} but by the activation of sBimR. Thus, sBimR was characterized to sense extracellular polyvalent cation concentration and to cause an increase in $Ca^{2+}{}_i$, which is observed as an increase in the Ca^{2+}-Cl^- current.

As expected from the high homology of the deduced amino acid of sBimR and mGluR, the oocytes injected with sBimR cRNA also responded to glutamate (Fig. 3a). The dose-response relationship of sBimR for glutamate was similar to that of the rat mGluR1α [4] (Fig. 3b).

To confirm that the induction of the Ca^{2+}-Cl^- current by the application of $Ca^{2+}{}_o$ or glutamate is due to an increase in the IP_3 turnover/$Ca^{2+}{}_i$ caused by the activation of the receptor, the following experiments were carried out. Injection of 10 nl of 10 mM EGTA, 10 min before the agonist application, abolished responses to both $Ca^{2+}{}_o$ and glutamate completely (data not shown), confirming that the Ca^{2+}-Cl^- current is caused by an increase of $[Ca^{2+}]_i$. Changes in the IP_3 turnover were also examined. When compared with the water injected oocytes, the sBimR cRNA injected oocytes showed an in-

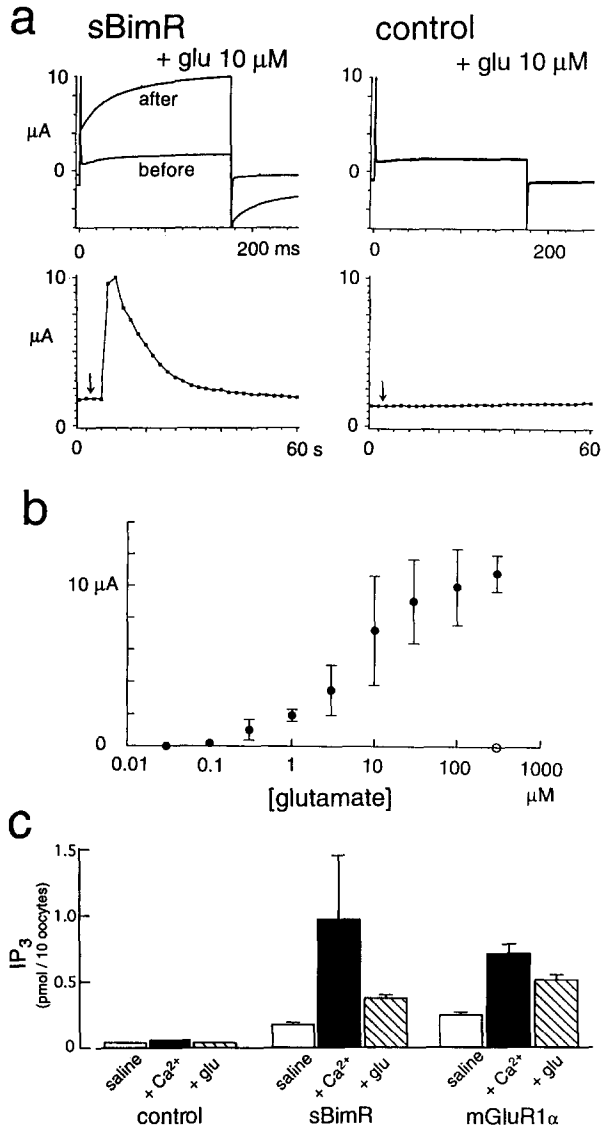

Fig. 3. Activation of Ca^{2+}-Cl^- current by glutamate in *Xenopus* oocytes injected with sBimR cRNA, and increased IP_3 turnover produced by extracellular Ca^{2+} or glutamate. (a) Ca^{2+}-Cl^- current of oocytes injected with sBimR cRNA induced by application of 10 μM glutamate to the bath solution (left, upper). The oocytes were put in the Ca^{2+} free saline solution, and then glutamate was added to the bath solution. Two current traces, before the application of glutamate, and at the peak of the induced response after the application, are shown (left, upper). The current amplitudes at the end of each depolarizing pulse are plotted to show the time dependence of the response. The time of application of glutamate is shown by the arrow (left, lower). When glutamate was applied to oocytes injected with water, no response was observed (right). (b) Dose-response curves of the Ca^{2+}-Cl^- current evoked by glutamate. Data for oocytes injected with sBimR cRNA (filled circles and error bars: mean and standard deviation, $n = 3$–7) or water (open circle and error bar: mean and standard deviation, $n = 3$; at 300 μM) are shown. (c) IP_3 levels of oocytes in the standard saline solution (open squares), in the saline solution containing 10 mM Ca^{2+} (filled squares) or 100 μM glutamate (hatched squares). Oocytes injected with water (left), sBimR cRNA (middle) or the rat mGluR1α cRNA (right) were examined. The measured values of the IP_3 level were 0.05, 0.11, 0.05, 0.35, 1.94, 0.74, 0.46, 1.42 and 0.99 (pmol/10 oocytes) from left to right. Error bars show standard deviations.

crease in IP_3 level by 18-fold or 15-fold after exposure to 10 mM Ca^{2+} or 100 μM glutamate, respectively (Fig. 3c). It is noteworthy that the basal level of IP_3 in the cRNA injected oocytes before the application of agonists was significantly higher than that of the water injected oocytes (Fig. 3c), presumably due to the 0.7 mM Ca^{2+} level in the bath solution. No significant increase in IP_3 was detected in the water injected oocytes exposed to $Ca^{2+}{}_o$ or glutamate (Fig. 3c). Thus, it was shown that sBimR is a bifunctional receptor which senses both extracellular Ca^{2+} and glutamate and is coupled to the turnover of IP_3 and the consequent increase in $[Ca^{2+}]_i$.

Using Northern blot analysis, a 5.5 kb band was detected in the brain but not in the heart, kidney, skeletal muscle, liver, testis or ovary (Fig. 4A). In situ hybridization analysis using a digoxigenin labeled probe showed the clearest expression of sBimR mRNA in cerebellar Purkinje cells (Fig. 4B). The observed distribution of sBimR mRNA is similar to that of the rat mGluR1α [4]. The similarity of the amino acid sequence and the pattern of distribution suggest that the sBimR is a salmon homologue of the rat mGluR1α.

To examine whether the rat mGluR1α also senses Ca^{2+} and other polyvalent cations or not, experiments similar to the above were performed. All the results: the dose response to $Ca^{2+}{}_o$ (Fig. 2c, open triangle), the order of preference for polyvalent cations (data not shown), the effect of EGTA injection (data not shown) and the increased turnover of IP_3 (Fig. 3c), closely resembled those for sBimR. Thus, it was shown that a bifunctional receptor for Ca^{2+} and glutamate is not specific to the salmon but is also present in mammalian brain, in the form of mGluR1α.

4. Discussion

In this work, a cDNA for sBimR which causes consistent activation of Ca^{2+}-Cl^- current without applying any special agonist was isolated from the salmon brain. sBimR was structurally highly similar to mGluR1α and weakly to CaR. It was functionally identified as a bifunctional G-protein couple type receptor which is activated by both $Ca^{2+}{}_o$ and glutamate. In addition, the rat mGluR1α was also shown to be activated by $Ca^{2+}{}_o$.

Brown et al. [10] and other investigators [9,11–13] have already reported structural similarity of CaR and mGluR1. The response of CaR to glutamate has already been tested [10], and it was shown that CaR senses only polyvalent cations but not glutamate. The response of the mGluR1α to $Ca^{2+}{}_o$, which we described in this report, has not been examined previously. In the previous study, the activation of the mGluR1α was monitored under voltage-clamp at hyperpolarized potential without giving step pulses as an increase in the inward current when glutamate was applied [4]. In this case, the high basal level of the Ca^{2+}-Cl^- current before glutamate application, induced by the $Ca^{2+}{}_o$ contained in the bath solution, could be observed only as a slight increase of the holding current. In contrast, we gave voltage step pulses repeatedly to depolarized potential where the channel is highly active. By this sensitive method, we noticed, for the first time, the increased basal level of Ca^{2+}-Cl^- current as a characteristic slowly activating and deactivating current (Fig. 2a).

The physiological significance of a metabotropic receptor for both extracellular $Ca^{2+}{}_o$ and glutamate in the brain could be as follows. (1) The amplitude of the response to glutamate might be modulated by the level of $[Ca^{2+}]_o$. The $[Ca^{2+}]_o$ level at the synaptic site is expected to be influenced not only by the

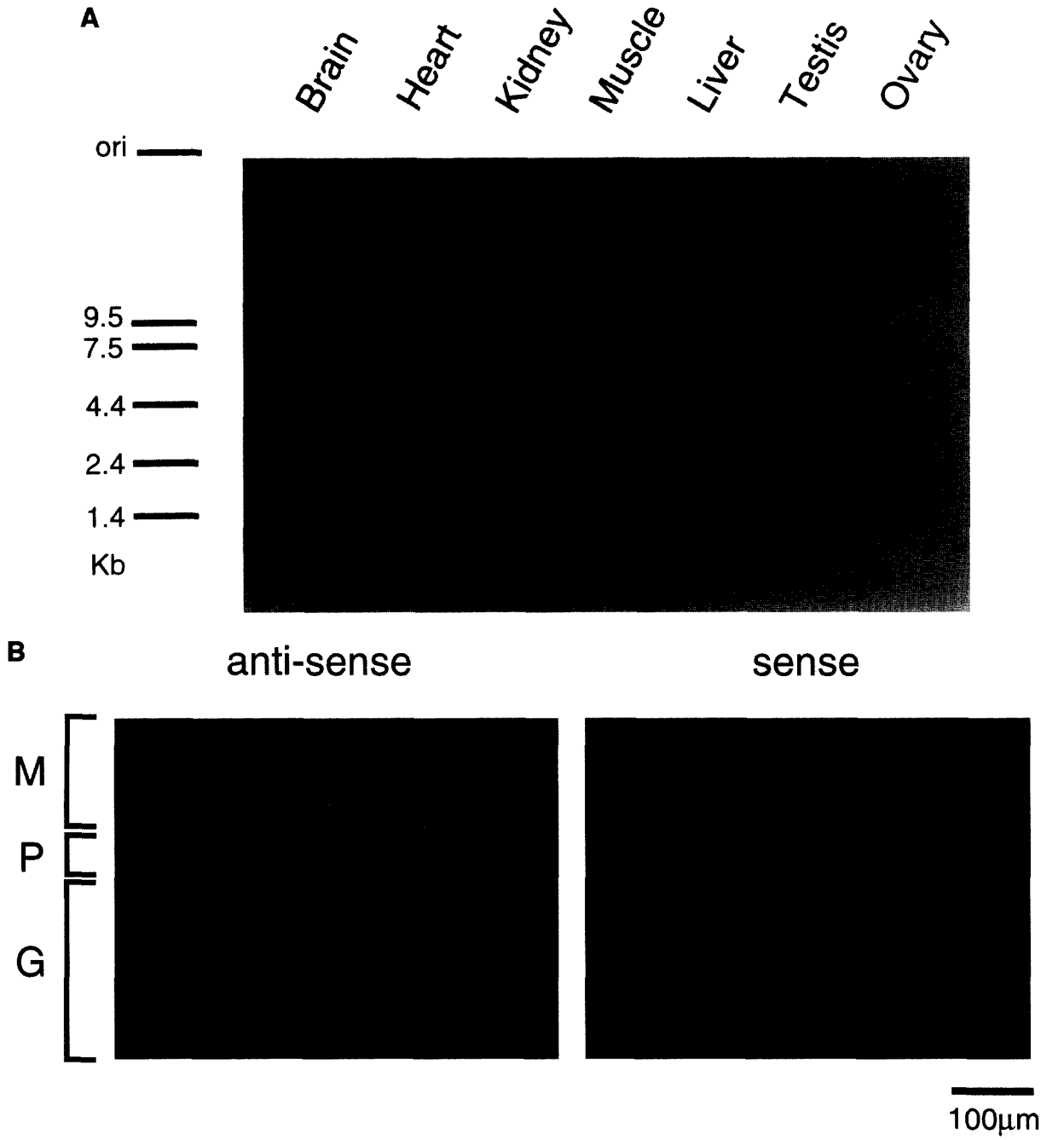

Fig. 4. The distribution of sBimR mRNA in various tissues and its localization within the salmon brain. (A) Analysis of sBimR mRNA distribution in various tissues by Northern hybridization. Expression was detected only in the brain. (B) Histochemical analysis of sBimR mRNA distribution in the brain by DIG-labeled in situ hybridization. The clearest expression was observed in the Purkinje cells in the cerebellum (left). 'M', 'P' and 'G' indicate molecular layer, Purkinje cell layer and granule cell layer of the cerebellum respectively. No clear signal was detected by a sense probe (right).

overall $[Ca^{2+}]_o$ level of the cerebrospinal fluid but also by the local regulation. For the local regulation, the $[Ca^{2+}]$ level of the synaptic vesicle which is co-released with glutamate and/or the uptake and release machineries of Ca^{2+} at the synaptic site might be critically important. (2) As neurons expressing this receptor are expected to receive continuous stimulation by $Ca^{2+}{}_o$ of the physiological range, their $IP_3/[Ca^{2+}]_i$ level might be basally high even when they are not excited. This persistent increase in $IP_3/[Ca^{2+}]_i$ might play important roles in various neuronal functions including plasticity. It is known that the mGluR1 antibody blocks the synaptic plasticity in the cerebellar slice [19]. The gene-targeted mice of the mGluR1α were reported to exhibit a deficient long-term potentiation in the hippocampus and abnormal spatial memory [20], and a deficient long-term depression in the cerebellum and impaired motor learning [21]. It is possible, for example, that the glutamate response of BimR is necessary for the acquisition of memory, and the consistent increase of $[Ca^{2+}]_i$ by $Ca^{2+}{}_o$ is required for the maintenance or the stabilization of memory.

Acknowledgements: We are grateful to Prof. S. Nakanishi for providing us with the rat mGluR1α cDNA; Dr. H.P.C. Robinson, Dr. S.

Ohsako and Dr. H. Okado for critical comments on the manuscript. This work was supported in part by research grants from the Mishima Kaiun Foundation (to K.K.), the Human Frontier Science Program (to Y.K.) and the Ministry of Education, Science, Sports and Culture of Japan (to K.K. and Y.K.).

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