FEBS Letters 533 (2003) 54–58 FEBS 26844

Cloning, tissue distribution and functional characterization of the chicken $P2X_1$ receptor

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Received 17 October 2002; accepted 7 November 2002

First published online 3 December 2002

Edited by Maurice Montal

Abstract We describe a new chicken P2X subunit that is an orthologue of the mammalian $P2X_1$ receptor. Functional characterization of chicken $P2X_1$ receptors was performed using the amphotericin B perforated patch configuration to avoid the current run-down observed under whole-cell patch-clamp conditions. Responses to agonists and to the antagonist PPADS (pyridoxal 5-phosphate 6-azophenyl-2′,4′-disulfonic acid) were similar to what has been described for mammalian orthologues. However, the antagonists suramin and NF023 were much less potent at chicken $P2X_1$ receptors than at human $P2X_1$ receptors. In embryonic tissues, transcript expression is predominant in lung, liver and skeletal muscle. Overlapping expression with $cP2X_4$ and $cP2X_5$ subunits in several embryonic tissues, including skeletal muscle, indicates that the native embryonic P2X receptors could be heteromultimeric.

Key words: ATP receptor; Perforated patch; Suramin; NF023; Skeletal muscle

1. Introduction

Extracellular ATP exerts its biological effects by binding to two different types of membrane receptors, P2X and P2Y receptors. P2X receptors are ligand-gated ion channels of which seven subunits have been cloned from mammalian tissues [1]. Several non-mammalian orthologues of P2X receptor subunits have been isolated and functionally characterized including zebrafish $P2X_3$ [2,3], zebrafish [4], chicken [5,6] and *Xenopus laevis* [7] $P2X_4$, zebrafish [4], chicken [8,9] and bull-frog [10] $P2X_5$ and *X. laevis* [11] $P2X_7$. In most cases, differences in the pharmacological and/or functional properties between mammalian and non-mammalian orthologue P2X receptors have been described.

$P2X_1$ receptor subunits were initially isolated from rat vas deferens by expression cloning [12]. The protein and mRNA show a wide distribution in rat tissues, although the highest levels of expression are found in smooth muscle containing tissues [13]. Indication that the native smooth muscle P2X receptor is mainly composed of $P2X_1$ subunits has been obtained by comparing the functional properties of native and heterologously expressed $P2X_1$ receptors [12,14] and by knocking out the $P2X_1$ gene [15]. $P2X_1$ subunit orthologues have been isolated from human urinary bladder and mouse vas deferens [16]. Homomeric mammalian $P2X_1$ receptors are rapidly desensitizing, α,β methylen-ATP (αβmeATP) sensitive receptors that are effectively blocked by suramin and suramin derivatives, pyridoxal 5-phosphate 6-azophenyl-2′,4′-disulfonic acid (PPADS) and trinitrophenyl-ATP (TNP-ATP) [16].

Responses to ATP have been demonstrated in embryonic skeletal muscle. Hume's group showed that ATP elicits a developmentally regulated depolarizing action on cultured chick myotubes [17]. There are indications that P2X receptors might also be involved in mammalian skeletal muscle development. Thus, $P2X_2$, $P2X_5$ and $P2X_6$ subunits are sequentially expressed in developing rat muscle [18] and responses to ATP have been reported in isolated mouse and human skeletal muscle cells during pre- and postnatal myogenesis [19,20]. In order to isolate P2X subunits from chicken embryos and to characterize their distribution and putative function during development, partial sequences of P2X receptor subunits expressed in chicken embryos were amplified using degenerate PCR. We have already described at the molecular level two chicken P2X receptor subunits: chicken $P2X_4$ ($cP2X_4$) [5] and chicken $P2X_5$ ($cP2X_5$) [9]. Both transcripts are present in developing skeletal muscle [6,9] and the $cP2X_5$ receptor is involved in the differentiation of satellite cells in mature muscle fibers [21]. Here we present the cloning and characterization of the third isolated chicken P2X subunit. The strong similarity with the already cloned $P2X_1$ subunits indicates that chicken $P2X_1$ ($cP2X_1$) is the first $P2X_1$ orthologue of non-mammalian origin. The overlapping expression with other P2X transcripts in several embryonic tissues including skeletal muscle strongly supports the presence of a heteromultimeric P2X receptor during development.

2. Materials and methods

2.1. Isolation of the $cP2X_1$ cDNA

A partial cDNA for the $cP2X_1$ subunit was obtained from chicken brain poly(A)$^+$ mRNA using degenerate PCR as previously described [22]. Specific sense (5′-GGACAGTTCTTTTGTGGTGATGAC-3′) and antisense (5′-GAAGGTGATGCTGTTCT TGATGAA-3′) oligonucleotides targeted to the known sequence of $cP2X_1$ were extended by PCR (Expand long template PCR system, Roche Biosciences, Palo Alto, CA, USA) towards the 5′ and 3′ mRNA ends. As templates, we

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Abbreviations: αβmeATP, α,β methylen-ATP; 2MeSATP, 2-methylthio-ATP; PPADS, pyridoxal 5-phosphate 6-azophenyl-2′,4′-disulfonic acid; NF023, 8,8′-(carbonylbis(imino-3,1-phenylene carbonylimino)bis(1,3,5-naphthalenetrisulfonic) acid)

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PII: S0014-5793(02)03751-1

used libraries of adaptor-ligated cDNA constructed following manufacturer's recommendations (Marathon cDNA amplification kit, BD Biosciences Clontech, Palo Alto, CA, USA) from 14-day-old chicken embryo skeletal muscle or heart poly(A)$^+$ mRNA. Fragments obtained from the PCR were cloned in pGEM-T (Promega, Madison, WI, USA) and sequenced. To obtain a full-length coding sequence, specific oligonucleotides were used to amplify an embryonic heart cDNA by PCR (PfuTurbo DNA polymerase, Stratagene, La Jolla, CA, USA). To avoid random mutations arising from the PCR, DNA fragments from independent reactions were cloned and sequenced.

2.2. Northern blot analysis

Poly(A)$^+$ mRNAs were isolated from tissues obtained from 15-day-old chicken embryos using the FastTrack Kit 2.0 (Invitrogen, Groningen, The Netherlands). The concentration of RNA in each sample was determined using the Ribogreen RNA quantitation kit (Molecular Probes, Leiden, The Netherlands). Ten micrograms poly(A)$^+$ RNA per tissue were separated in a 1% agarose gel containing formaldehyde, capillary transferred onto a positively charged nylon membrane (Roche Biosciences, Palo Alto, CA, USA) and UV-crosslinked. The membrane was hybridized with a [α-^{32}P]dCTP labeled probe containing the complete coding region of cP2X$_1$. The hybridization was performed in ExpressHyb solution (Clontech, Palo Alto, CA, USA) at 68°C for 1 h, the final wash was done in 0.1×SSC, 0.1×SDS at 65°C. The membrane was exposed to an X-ray film for three days.

2.3. Electrophysiological recordings

The full-length cP2X$_1$ cDNA was subcloned into the mammalian expression vector pcDNA3 (Invitrogen, Groningen, The Netherlands). Chinese hamster ovary (CHO) cells were transfected, selected using 1 mg ml^{-1} G418 (Sigma, St. Louis, MO, USA), cloned and maintained using 0.5 mg ml^{-1} G418 as previously described [23].

For electrophysiological measurements, both the amphotericin B perforated patch configuration [24] and the whole-cell configuration [25] of the patch-clamp technique were used. Recordings were made with an EPC-9 amplifier and Pulse+Pulsefit software package (HEKA Elektronics, Lambrecht, Germany). All measurements were carried out at room temperature. Pipette solution for perforated patch recordings contained (in mM): 120 K-gluconate, 20 KCl, 10 BAPTA, 10 HEPES, pH 7.2. Thin borosilicate pipettes (2.5–3 MΩ) were backfilled with the same solution containing 300 μg ml^{-1} amphotericin B (Sigma, St. Louis, MO, USA; stock solution in DMSO, final DMSO dilution 0.6%). For whole-cell recordings we used identical pipettes filled with a solution containing (in mM): 120 K-gluconate, 20 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 5 mM EGTA, 10 mM HEPES, pH 7.2. Extracellular solution contained (in mM): 140 NaCl, 2.8 KCl, 2 $CaCl_2$, 10 HEPES, pH 7.2. Currents were low-pass filtered at 500 Hz and digitized at 2 kHz. Typical holding potential was −70 mV. Agonists dissolved at the appropriate concentration were rapidly applied using a DAD-12 application system (ALA Scientific Instruments, Westbury, NY, USA). Antagonists were superfused for 5–10 min before being applied together with the agonist. All data points are mean ± S.E.M. of n determinations.

2.4. Drugs

ATP, αβmeATP, β,γ methylen-ATP (βγmeATP), UTP and suramin were purchased from Sigma (St. Louis, MO, USA). 2-Methylthio-ATP (2MeSATP) and PPADS were obtained from Tocris Cookson (Bristol, UK). 8,8′-(Carbonylbis(imino-3,1-phenylene carbonylimino)-bis(1,3,5-naphthalenetrisulfonic) acid) (NF023) was obtained from Calbiochem (San Diego, CA, USA).

3. Results and discussion

PCR with degenerate oligonucleotides obtained from the sequences of the known P2X receptors was used to PCR cDNA obtained from chicken brain. A partial sequence of approximately 740 bp was obtained and this sequence information was used to clone a full-length cDNA from embryonic chicken skeletal muscle and heart by 3′ and 5′ RACE-PCR. The obtained cDNA is 1610 bp long, contains an open reading frame preceded by a stop codon and encodes a protein of 392 amino acids (Fig. 1) with a calculated molecular weight of 44.5 kDa. This protein is around 65% identical to both rat and human P2X$_1$ [12,26] with significantly lower sequence identity to other P2X receptor subunits, suggesting it is the chicken orthologue of P2X$_1$ (cP2X$_1$). The identity is well distributed along the sequence except for the last amino acids of the carboxy terminus (Fig. 1). Differences in the primary sequence of the carboxy terminal domain between orthologues have also been described for human, rat and chicken P2X$_5$ subunits [9]. cP2X$_1$ presents the two putative transmembrane domains and the 10 cysteines in the extracellular loop that are conserved for all P2X receptors. The disruption of disulfide bonds between cysteines by site-directed mutagenesis affects the trafficking of human P2X$_1$ receptor subunits to the plasma

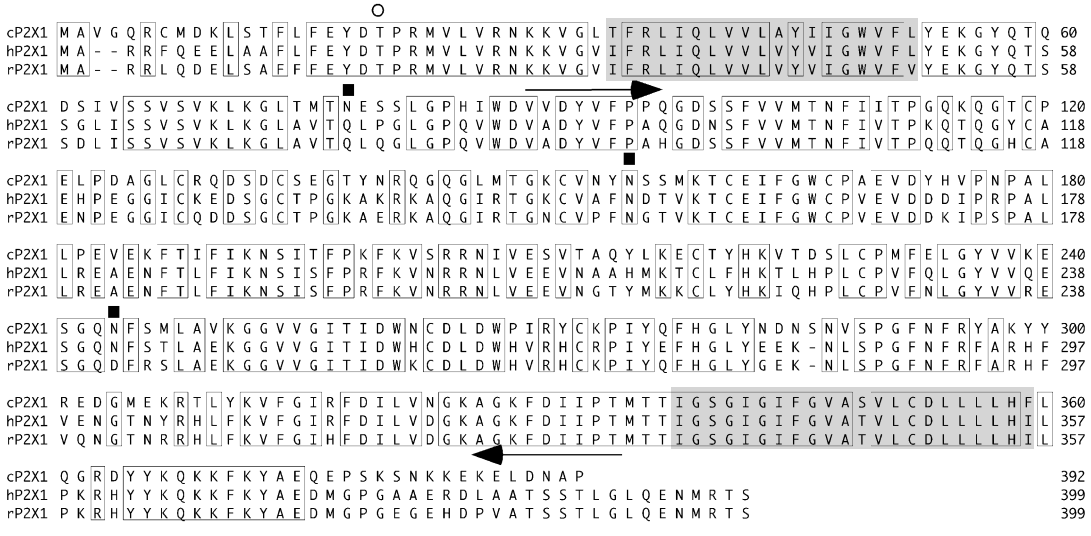


Fig. 1. Alignment of chicken, human and rat P2X$_1$ receptor subunits. Residues identical in all sequences are denoted by boxes. Squares: putative N-glycosylation sites [N-X-(S/T)]; circle: putative PKC phosphorylation site conserved in all P2X subunits. Shaded boxes mark the transmembrane domains and the arrows indicate the position of the degenerate oligonucleotides used to isolate cP2X$_1$. The sequence is in GenBank, accession number AJ511278.

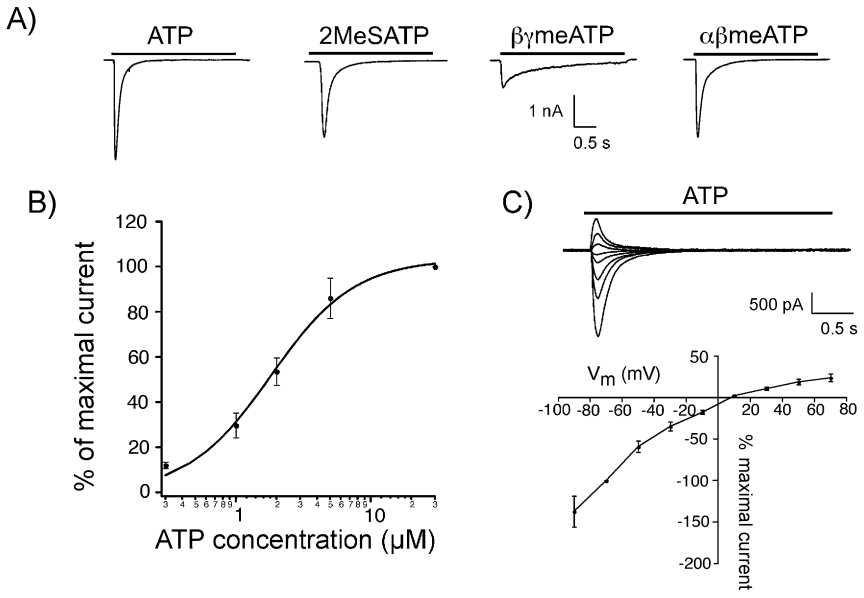
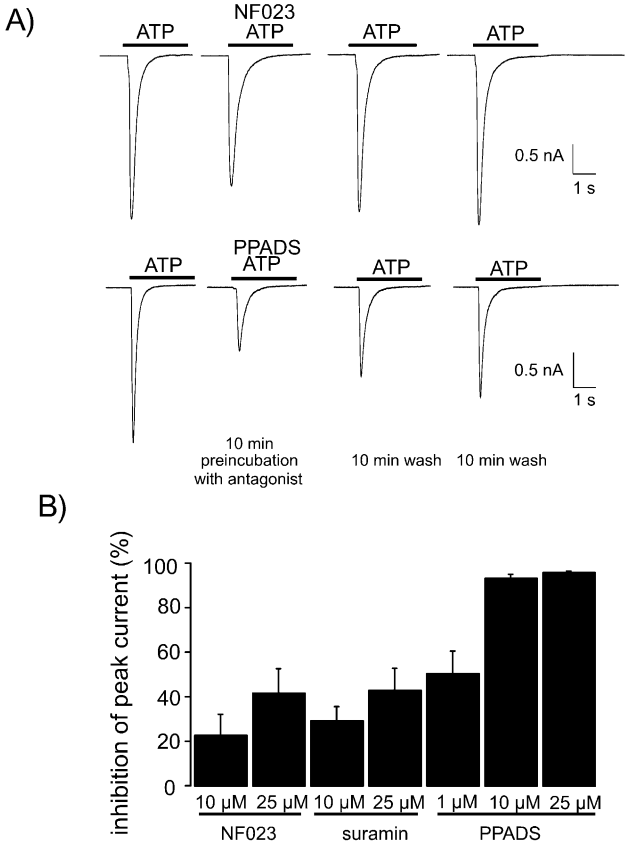


Fig. 2. Agonist profile of recombinant chicken $P2X_1$ receptors. A: Whole-cell currents measured in the perforated patch configuration in response to stimulation of $cP2X_1$ receptors by various nucleotides at a concentration of 30 μM. The holding potential was −70 mV. B: Dose–response curve for ATP. The continuous line for ATP is the fit to the data using the equation $I = I_{max}/(1+(EC_{50}/L)^{nH})$, where I is the actual current for a ligand concentration (L), nH is the Hill coefficient and I_{max} is the maximal current: $EC_{50} = 1.8 \pm 0.2$ μM, with a Hill coefficient of 1.4 ± 0.2 ($n = 3$–6). The amount of current obtained at each ATP concentration was normalized to the current obtained with 30 μMATP. C: maximal currents elicited by 30μM ATP at different holding voltages ($n = 3$). I–V steady state curve showing inward rectification. Reversal potential after correction for liquid junction potentials was -0.6 ± 0.9 mV.



membrane [27], indicating their importance for correct processing of the protein. Additionally, three putative *N*-linked glycosylation sites are present in the primary sequence (N78, N155, N244), two of them (N155 and N244) are also present in the $hP2X_1$ sequence while only one putative *N*-glycosylation position (N155) is coincident with one of the four *N*-glycosylated sites in the $rP2X_1$ protein. Any two of those four putative *N*-glycosylation sites must be glycosylated to ensure fully functional expression of $rP2X_1$ receptors [28], explaining the lack of conservation of putative *N*-glycosylated positions between orthologues.

To characterize the pharmacological and functional properties of $cP2X_1$ receptors we transfected CHO cells with the $cP2X_1$ subunit. ATP evoked currents (1.4 ± 0.2 nA for 10 μM ATP, $n = 26$) that desensitized in the presence of the agonist with a time constant of 203.9 ± 9.9 ms ($n = 20$). Following repeated applications at 8-min intervals in the whole-cell recording configuration the peak currents declined, longer washing intervals (up to 30 min) had no effect on the recovery of the current (not shown). Run-down of the current most likely results from the dialysis of the cell with the pipette solution leading to the dilution of intracellular components. The same phenomenon has been shown for native P2X receptors in smooth muscle tissue and could be avoided by performing

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Fig. 3. Antagonist profile of recombinant chicken $P2X_1$ receptors. A: Whole-cell currents measured in the perforated patch configuration upon application of 10 μM ATP in the absence and in the presence of 10 μM NF023 or 1 μM PPADS. Both antagonist were pre-applied for 10 min. The interval between ATP applications was 10 min. B: Percentage of block of the current elicited by 10 μM ATP in the presence of different concentrations of the antagonists suramin, NF023 or PPADS.

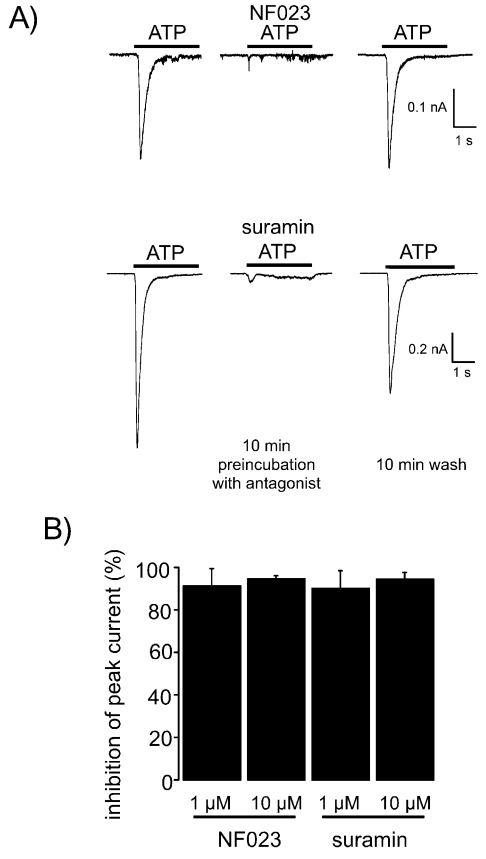

Fig. 4. Antagonist profile of recombinant human $P2X_1$ receptors. A: Whole-cell currents measured in the perforated patch configuration upon application of 10 μM ATP in the absence and in the presence of 10 μM NF023 or suramin. Both antagonists were pre-applied for 10 min. The interval between ATP applications was 10 min. B: Percentage of block of the current elicited by 10 μM ATP in the presence of different concentrations of the antagonists suramin and NF023.

the measurements in the perforated patch configuration of the patch-clamp technique [29]. In fact, using the perforated patch configuration we obtained reproducible responses by repetitive applications of agonists at 8–10-min intervals. Representative currents obtained upon application of agonists are shown in Fig. 2A. The rank order of agonist efficiency (at 30 μM) was: ATP > BzBzATP > 2MeSATP = α,βmeATP > β,γ meATP > UTP ($n = 3$–7). Construction of a dose–response curve for ATP gave an EC_{50} value of 1.8 ± 0.2 μM, with a Hill coefficient of 1.4 ± 0.2 ($n = 3$–6) (Fig. 2B). The current–voltage relationship for ATP-evoked P2X receptor currents (Fig. 2C) was inwardly rectifying and reversed at -0.6 ± 0.9 mV after correcting for liquid junction potentials [30]. Additionally, we analyzed the sensitivity of $cP2X_1$ to several P2 antagonists including suramin, NF023 and PPADS (Fig. 3). Surprisingly, both suramin and NF023 were poor antagonists at $cP2X_1$ receptors compared to what has been described for the mammalian orthologues [12,14,31]. Thus, 10 μM suramin or NF023 blocked only $29.4 \pm 6.2\%$ ($n = 11$) or $22.8 \pm 9.3\%$ ($n = 13$) of the current evoked by 10 μM ATP (Fig. 3A,B). Increasing the concentration of suramin and NF023 to 25 μM produced a blockade of the current of $43.1 \pm 9.7\%$ ($n = 13$) and $41.8 \pm 10.8\%$ ($n = 11$), respectively. In order to compare this data with the data obtained at mammalian orthologue receptors under our measuring conditions, HEK cells transfected with human $P2X_1$ were measured. Suramin and NF023 were effective blockers of human $P2X_1$ receptors (Fig. 4), with 1 μM of suramin or NF023 blocking approximately 90% of the current elicited by 10 μM ATP (91.6 ± 7.8 for NF023, 90.5 ± 7.8 for suramin, $n = 4$). Human $P2X_1$ receptors with altered sensitivity to suramin have been obtained upon mutation of several positively charged amino acids located at the extracellular domain of the protein [32]. However, all five amino acid residues (K70, K215, K309, R202 and R292 of $hP2X_1$) are conserved between human and chicken $P2X_1$ subunits. In contrast, PPADS was an effective antagonist at $cP2X_1$ receptors ($IC_{50} \approx 1$ μM, $n = 5$) (Fig. 3A,B) in a comparable way to what has been described for $hP2X_1$ receptors [14].

Northern blot hybridization analysis of mRNA isolated from several chicken embryonic tissues (15-day-old embryos) revealed the expression of a predominant transcript of 1.95 kb with two more transcripts (2.2 and 2.8 kb) showing lower abundance (Fig. 5). The highest level of expression was found in lung. Transcripts were also detected in skeletal muscle, liver and heart. Presence of transcripts of different length has also been observed for rat $P2X_1$ and has been attributed both to the presence of 3′ untranslated region of different length and to the presence of incompletely spliced mRNA [12]. By Northern blot and in situ hybridization, $cP2X_4$ and $cP2X_5$ transcripts were also found to be present in skeletal muscle, with further overlapping expression in other embryonic tissues (including heart and lung) [5,6,8,9,33]. Co-expression of rat $P2X_1$ and $P2X_5$ subunits produced a heteromeric receptor with specific functional properties [34,35]. Moreover, heteromultimerization between $rP2X_4$ and $rP2X_5$ subunits has been shown by co-immunoprecipitation although no functional

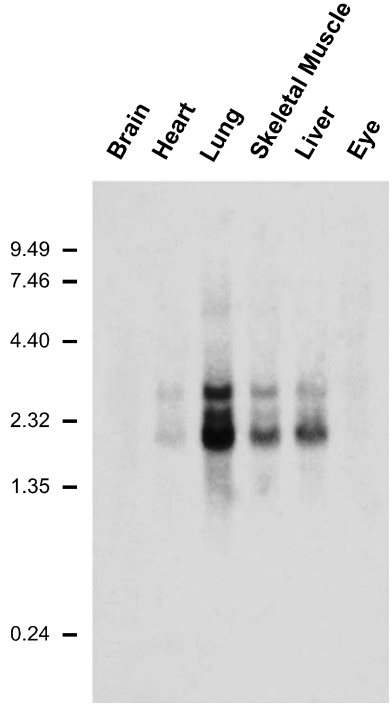

Fig. 5. Chicken $P2X_1$ transcript distribution in several chicken embryonic tissues. Ten micrograms poly(A)$^+$ mRNA were used per lane. The length of the $cP2X_1$ transcript is approximately 1.9 kb in all tissues analyzed.

characterization has been described so far [36]. Homomeric $cP2X_5$ receptors present most of the functional properties observed in native skeletal muscle P2X receptors with the exception of the response to αβmeATP [9,37]. Most likely the native skeletal muscle P2X receptor in chicken is a heteromultimeric receptor that might be composed of $P2X_1$, $P2X_4$ and $P2X_5$ subunits.

In summary, we have isolated a new P2X subunit that is the chicken orthologue of mammalian $P2X_1$ receptors. Run-down of the current elicited by ATP at $cP2X_1$ receptors could be avoided by using the perforated patch configuration of the patch-clamp technique. Agonists sensitivity was similar to mammalian $P2X_1$ receptors. However, $cP2X_1$ receptors were less sensitive to suramin and suramin analogs than the mammalian counterparts. The presence of $cP2X_1$ transcripts in skeletal muscle of chicken embryos could indicate a possible heteromultimerization with the $cP2X_5$ and the $cP2X_4$ subunit.

Acknowledgements: We are indebted to Walter Stühmer for generous support. We thank Fritz Benseler and Ivonne Thanhäuser for performing the DNA sequencing and Ludwig Kolb for graphic work. We are grateful to Daniel Kerschensteiner for suggestions and for critically reading the manuscript.

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