Ca^{2+} Signals Mediated by P2X-Type Purinoceptors in Cultured Cerebellar Purkinje Cells

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We have studied $[Ca^{2+}]_i$ signals elicited by extracellular ATP in cultured cells from postnatal day 7–8 rat cerebellum using single-cell fluorescence microscopy and fura-2. Putative Purkinje cells selected under phase contrast by size and characteristic cytoplasm appearance were uniquely identified by selective labeling with anti-calbindin antibodies. Extracellularly applied ATP (50 μM) evoked fast $[Ca^{2+}]_i$ rises revealed by a rapid and transient increase in fura-2 F_{340}/F_{380} ratio in all Purkinje cells tested, whereas granule cells failed to show a response to ATP. The mean $[Ca^{2+}]_i$ increase was ~400 nM, comparable to that obtained after glutamate stimulation. The response to ATP was completely abolished by removal of extracellular Ca^{2+} with EGTA. Conversely, an increased extracellular Mn^{2+} entry pathway was activated by ATP stimulation. These results indicate that the effect of ATP is mediated by an ionotropic P2X receptor. The action of ATP was mimicked by the analog 2-methylthio-adenosine 5′-triphosphate with similar efficacy but almost half its potency (EC_{50}, 10.6 ± 0.7 vs 21.7 ± 1.9 μM). Other purinergic compounds tested, such as adenosine(5′)-tetraphospho-(5′)adenosine, adenosine(5′)-pentaphospho-(5′)adenosine, adenosine 5′-(α,β-methylene) triphosphate, UTP, and adenosine, were completely inactive in eliciting $[Ca^{2+}]_i$ responses. The purinoceptor antagonists suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid effectively blocked the responses elicited by ATP. Our results demonstrate for the first time the presence of functional ionotropic P2X purinoceptors in the cerebellar Purkinje cells and indicate that their pharmacology is similar to receptors formed by $P2X_2$ subunit oligomers.

Key words: ATP; ATP receptors; Purkinje cells; cell culture; fura-2 microfluorescence; suramin; PPADS; cerebellum; rat

The role of ATP as an extracellular neurotransmitter at peripheral neural and non-neural systems is now well established (Dubyak and El-Moatassim, 1993). On the contrary, the actions of extracellular ATP on central neurons remain largely uninvestigated. Despite early evidence implicating ATP as a possible transmitter at central synapses (Phillis and Wu, 1981; Jahr and Jessel, 1983), only after the demonstration of excitatory postsynaptic events mediated by ATP in cultured neurons from rat coeliac ganglion (Evans et al., 1992; Silinsky et al., 1992) and rat medial habenula slices (Edwards et al., 1992), the ATP has been recognized as a fast synaptic transmitter in the CNS. As a consequence, an increasing number of actions evoked by extracellular ATP have been found throughout the brain, including the ability of ATP to activate inward currents in neurons of locus coeruleus (Harms et al., 1992; Shen and North, 1993) and spinal dorsal horn (Li and Perl, 1995) and to evoke extracellular Ca^{2+} entry in spinal (Salter and Hicks, 1994) and hypothalamic neurons (Chen et al., 1994). The ATP-induced fast excitatory responses have pharmacological and electrophysiological characteristics indicating the involvement of P2X-type ionotropic purinoceptors rather than metabotropic P2Y purinoceptors (Surprenant et al., 1995). The P2X receptor family, structurally distinct from other ionotropic receptors, comprises seven subunit types cloned so far. These subunits can be grouped according to the pharmacological profile and inactivation kinetics of homomeric receptors formed by them (Lewis et al., 1995; Buell et al., 1996a).

The autoradiographic identification of ATP binding sites using the nonhydrolyzable analog [^{3}H]adenosine 5′-(α,β-methylene) triphosphate ([^{3}H]α,β-meATP) reveals a widespread distribution across the brain, with cerebellar cortex showing the highest levels of binding (Bo and Burnstock, 1994; Balcar et al., 1995). Similarly, *in situ* hybridization studies have demonstrated the presence of P2X mRNAs through the brain, especially in cerebellum, hippocampus, and olfactory bulb, for all members of the family (Bo et al., 1995; Buell et al., 1996b; Collo et al., 1996; Séguéla et al., 1996; Soto et al., 1996), except for the $P2X_3$ subunits that are restricted to dorsal root ganglia (Chen et al., 1995) and $P2X_5$ subunits that are expressed in dorsal root ganglia and spinal cord but apparently not in brain, except for neurons in the mesencephalic trigeminal nucleus (Collo et al., 1996). $P2X_4$ and $P2X_6$ receptor subunits are the members of this family most highly expressed in central neurons. Heterologously expressed $P2X_4$ and $P2X_6$ receptors produced nondesensitizing inward currents activated by ATP and 2-methylthio-adenosine 5′-triphosphate (2MeSATP) but not by α,β-meATP. A distinguishing characteristic of the $P2X_4$ and $P2X_6$ receptors is the lack of sensitivity to antagonists such as suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), which do not block, or even potentiate, the responses to ATP mediated by them in heterologous expression systems (Bo et al., 1995; Buell et al., 1996b; Collo et al., 1996). Because the effects of ATP on central neurons found so far appear to be antagonized effectively by suramin, we decided to study the functional responses to ATP in cerebellar Purkinje

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cells, which show the highest levels of expression of $P2X_4$ and $P2X_6$ receptors in the brain, and their sensitivity to suramin as a purinergic blocker.

MATERIALS AND METHODS

Cultures of rat cerebellar neurons. Cerebellar cells were obtained from 7- to 8-d-old Wistar rats. Groups of 8–10 cerebella were pooled in isolation buffer consisting of (in mM): NaCl, 130; KCl, 4; Na_2HPO_4, 10; $MgSO_4$, 1.5; HEPES (acid), 10; glucose, 15; and bovine serum albumin (BSA), 0.05; pH 7.4, plus penicillin (50 U/ml) and streptomycin (50 μg/ml). The cerebella were then cut into small pieces (~500 μm thick), dispersed into 10 ml of isolation buffer supplemented with 0.25 mg/ml trypsin (Sigma, Alcobendas, Spain), and incubated for 20 min at 37°C with a gently stirring every 5 min. The trypsinization was stopped by addition of 10 ml of isolation buffer containing 0.25 mg/ml soybean trypsin inhibitor (Sigma) and 10 U/ml DNase I (Sigma) and centrifuged at 65 × *g* for 1 min. The supernatant was discarded, and the pellet was triturated by passing it through a fire-polished Pasteur pipette in 2 ml of isolation buffer supplemented with 1.5 mM $MgSO_4$, 0.25 mg/ml soybean trypsin inhibitor, and 250 U/ml DNase I. The triturating process was followed until no material could be seen in suspension. Perikarya were separated on a self-generated Percoll (Pharmacia, Madrid, Spain) gradient containing 20 ml of isotonic Percoll (16 ml of Percoll plus 4 ml of fivefold-concentrated isolation buffer) plus 20 ml of cell suspension. The mixture was centrifuged at 20,000 × *g* for 30 min at 22°C. In these conditions two clear fractions could be distinguished. The upper fraction (corresponding to lower densities) contained ~50% of the total number of cells centrifuged, as well as all the debris and nonperikarya material. The lower fraction (higher densities) was composed only of perikarya, mainly of granule cells, but also a small number of large-size cells could be observed by phase-contrast microscopy. We also obtained cultures by the method described by Dutton et al. (1981) in which the separation of the perikarya is performed in a gradient of 4% BSA and centrifugation at 100 × *g* for 5 min. These cultures contained more debris than the Percoll ones, and large-size cells were scarcely found. Therefore, Percoll gradients produced more clean preparations and a relative enrichment in large-size cells (putative Purkinje neurons).

Thus, the lower fraction of the Percoll gradient was collected and washed two times by a 5× dilution with isolation buffer and centrifugation at 150 × *g* for 10 min. The pellet was resuspended in DMEM (Life Technologies, Barcelona, Spain) containing 10% (v/v) fetal calf serum (Life Technologies), 30 mM glucose, 50 U/ml penicillin, 50 μg/ml streptomycin, 100 μg/ml kanamycin, and 2.5 μg/ml amphotericin, at a density of 10^6 cells/ml, and the cells were then plated at 500,000/cm^2 over 15-mm-diameter poly-L-lysine-coated coverslips. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Medium was replaced every 4 d. The experiments were performed during the first 5 d *in vitro*.

Intracellular Ca^{2+} measurements. The $[Ca^{2+}]_i$ was recorded from single neurons essentially as described by Mateo et al. (1996) using a multiple excitation microfluorescence system (Cairn Research Ltd., Kent, UK). Cells attached to coverslips were washed in Locke's solution [in mM: NaCl, 140; KCl, 4.5; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; glucose, 5.5; and HEPES (acid), 10; pH 7.4] supplemented with 1 mg/ml BSA. The cells were then loaded with 5 μM fura-2 AM (Molecular Probes, Eugene, OR) for 45 min at 37°C. During loading and further washing, the cells were exposed to ATP-free Locke's medium for at least 1 hr. The coverslip was placed in a small superfusion chamber on the stage of a Nikon Diaphot microscope, and the cells were illuminated alternately at 340, 360, and 380 nm through a 100× 1.3 numerical aperture objective. The emitted fluorescence was driven to the photomultiplier after passing through a 510 nm bandpass interference filter. The F_{340}, F_{360}, and F_{380} signals were acquired at 128 Hz, filtered, and digitized at 4 Hz. The measuring field was routinely centered on the cell of interest by means of a rectangular diaphragm placed on the emission path blocking all incoming light but that from the selected cell. $[Ca^{2+}]_i$ was derived from the F_{340}/F_{380} ratio using the equation derived by Grynkiewicz et al. (1985) and R_{max} and R_{min} parameters from an *in vitro* calibration. The F_{360} signal (isosbestic point, Ca^{2+}-insensitive) was monitored as an internal control. The *in vitro* calibration of the photometric system was performed by recording fluorescence from small droplets of fura-2 (free acid, Molecular Probes) dissolved in intracellular solution (in mM: 100 KCl, 10 NaCl, 1 $MgCl_2$, 10 MOPS, and 1–2 μM fura-2, pH 7.0) plus 2.5 mM $CaCl_2$ (saturated Ca^{2+}) or 2.5 mM EGTA (zero Ca^{2+}). This procedure gives only approximate $[Ca^{2+}]_i$. A correction factor to compensate for differences in R_{max} and R_{min} parameters between calibration solutions and cell cytosol was calculated accordingly to the procedure of Poenie (1991), by measuring the $\Delta F_{340}/\Delta F_{380}$ ratio for fast changes in $[Ca^{2+}]_i$ in each cell (F_{360} was confirmed to remain constant).

Superfusion and drug application. The superfusion chamber was carved in a Perspex block and closed with the coverslip containing the cells forming the bottom of a 34 μl space. The medium was fed by gravity at 1–1.5 ml/min from a prewarmed reservoir and continuously aspirated from the chamber outlet. The temperature was adjusted to 30°C. Drugs were applied to the cells by switching the superfusion solution with the aid of a four-way stopcock. The chamber worked in a plug-flow way; changing of chamber solution was complete within 1 sec. In addition, this rapid flow prevented the accumulation of any compound (ATP or glutamate), secreted by the cells or released on cell death, inside the chamber. UTP was from Boehringer Mannheim (Barcelona, Spain). ATP, 2MeSATP, adenosine(5′)-tetraphospho-(5′)adenosine (Ap_4A), and suramin were obtained from Research Biochemicals (Natick, MA). Adenosine, α,β-meATP, adenosine(5′)-pentaphospho-(5′)adenosine (Ap_5A), and L-glutamic acid were from Sigma.

Immunocytochemical identification of Purkinje neurons and glia. For differential identification of Purkinje neurons and glia, selective staining for calbindin D-28K and glial fibrillary acidic protein (GFAP) were performed by immunocytochemistry in the same coverslips as those used for $[Ca^{2+}]_i$ measurements. Once the $[Ca^{2+}]_i$ experiments had been performed, the cells were fixed with 50% acetone/50% methanol (v/v) for 1–2 min at 4°C, followed by freezing and storage at −20°C until processing. For the immunofluorescence assay, the fixed coverslips were thawed by placing them in ice-cold 50% acetone/50% methanol and warmed to room temperature. The preparation was rehydrated in PBS and blocked with 3% BSA/0.1% Triton X-100 in PBS. Primary antibodies (mouse monoclonal anti-calbindin D-28K, 1:100; and rabbit polyclonal anti-GFAP, 1:200) were incubated for 1 hr. Excess of antibodies was washed three times in blocking medium. Labeling was revealed with goat anti-mouse IgG FITC-conjugated and goat anti-rabbit IgG tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibodies (1:200, 1 hr incubation). All antibodies were obtained from Sigma. The coverslips were viewed with a Nikon Diaphot microscope equipped with a 40× phase-contrast objective and fluorescein–rhodamine Nikon filter sets. Images were acquired and digitized at 256 gray levels.

RESULTS

Cell identification

We used a combination of morphological and functional criteria to identify cells in the cultures. Morphologically, the Purkinje-like cells could be identified easily by their large size (~15 μm), a characteristic homogeneously granular cytoplasm appearance, and their round shape. Figure 1*A* shows examples of putative Purkinje cells (*arrows*) viewed through phase-contrast optics. To confirm that these large cells were indeed Purkinje neurons (rather than, for instance, Golgi cells or astrocytes), we performed double immunostaining with anti-calbindin and anti-GFAP antibodies. As can be seen in Figure 1*B*, anti-calbindin antibodies specifically and selectively labeled putative Purkinje cells identified in phase contrast. The very abundant, small, ovoid, and compact cells extending long neurites were regarded as granule cells. Calbindin-positive Purkinje neurons were more abundant in the first 2 d in culture, and their number declined rapidly thereafter during the first week. At 7 d *in vitro* they were scarcely found. During this period the Purkinje cells remained round, without extending processes, in agreement with other studies showing that Purkinje cells from animals older than 2–3 d lack the ability to outgrow neurites but retain a round shape for up to 30 d *in vitro* (Hockberger et al., 1989). Our cultures also contained other cells types, including astrocytes (identified in Fig. 1*C* with anti-GFAP labeling) and nongranule, non-Purkinje neurons in a minor proportion.

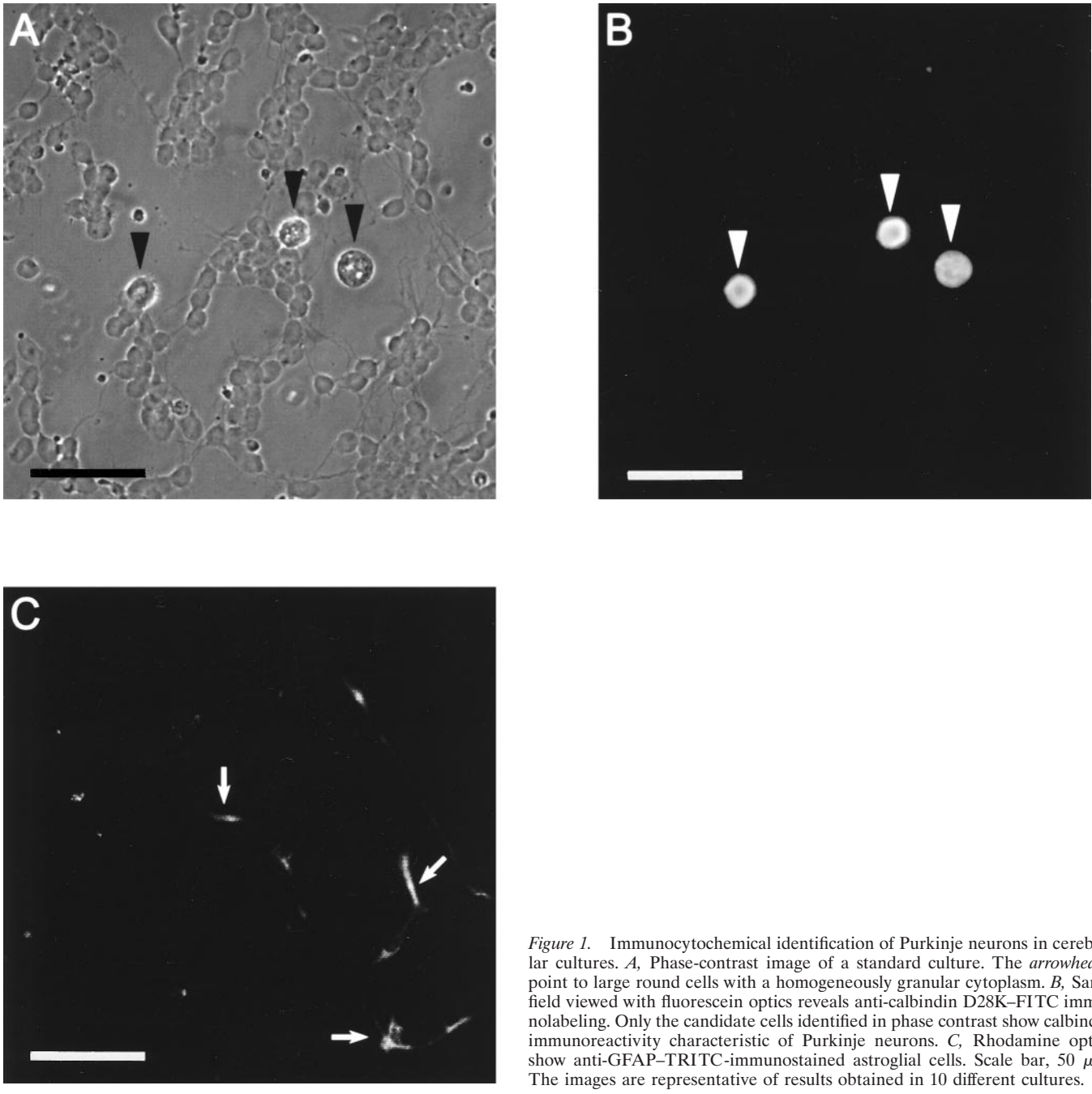

Figure 1. Immunocytochemical identification of Purkinje neurons in cerebellar cultures. *A,* Phase-contrast image of a standard culture. The *arrowheads* point to large round cells with a homogeneously granular cytoplasm. *B,* Same field viewed with fluorescein optics reveals anti-calbindin D28K–FITC immunolabeling. Only the candidate cells identified in phase contrast show calbindin immunoreactivity characteristic of Purkinje neurons. *C,* Rhodamine optics show anti-GFAP–TRITC-immunostained astroglial cells. Scale bar, 50 μm. The images are representative of results obtained in 10 different cultures.

ATP-activated $[Ca^{2+}]_i$ signals in Purkinje cells

Purkinje neurons identified as described above were challenged with glutamate and ATP to elicit $[Ca^{2+}]_i$ signals. As shown in Figure 2, both compounds evoked rapidly rising $[Ca^{2+}]_i$ transients detected by fura-2. The $[Ca^{2+}]_i$-increasing effect of ATP was unchanged throughout the first 5 d in culture, although the total number of Purkinje neurons decreased rapidly. However, the $[Ca^{2+}]_i$ increase induced by ATP was highly variable from cell to cell in the same coverslip, as was the response to glutamate. The basal $[Ca^{2+}]_i$ level was 113 ± 6 nM, and the average $[Ca^{2+}]_i$ increase after 50 μM ATP amounted 390 ± 50 nM (n = 51 cells), which represent 66% of average 100 μM glutamate-evoked peak $[Ca^{2+}]_i$ rises. The ATP-elicited response declined gradually toward basal levels in the continuous presence of the agonist, indicating receptor desensitization. Glutamate evoked a clear $[Ca^{2+}]_i$ rise even after the complete desensitization of ATP response. Furthermore, ATP was able to elicit a $[Ca^{2+}]_i$ increase on top of a glutamate challenge, indicating that these two agonists were acting on different receptors.

The action of ATP was totally dependent on the presence of extracellular Ca^{2+} (Fig. 3, *cell 1*). Reduction of extracellular $[Ca^{2+}]$ to ~200 nM with an EGTA buffer reduced the ATP response by 97.3 ± 1.6% (n = 15 cells). This effect was specific for Purkinje cells, because the ATP-elicited $[Ca^{2+}]_i$ increase in neighboring astrocytes in the same coverslip was not affected by the presence of EGTA (Fig. 3, *cell 2*). Nevertheless, the abolishing of the ATP effect in Purkinje cells by EGTA could also arise from a depletion of intracellular stores of Ca^{2+} by the EGTA pretreat-

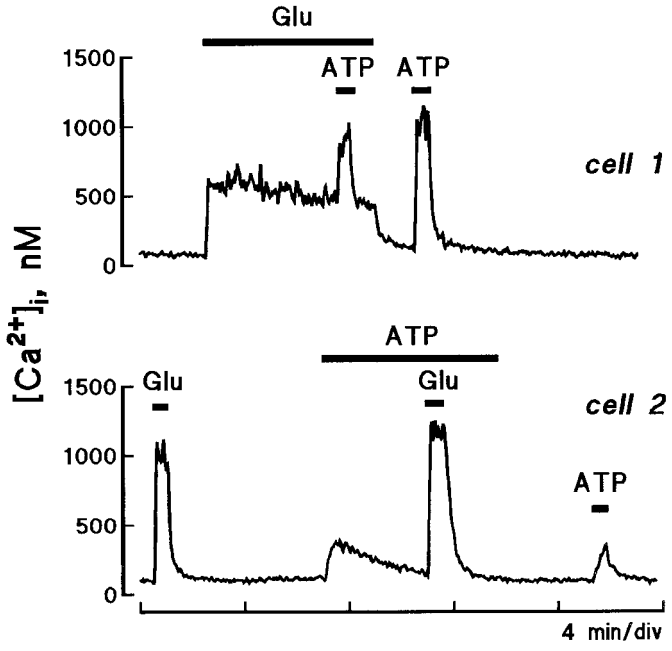

Figure 2. Ca^{2+} responses evoked by glutamate and ATP in single Purkinje neurons. The cells were continuously perfused with fresh medium and drugs applied with the superfusion flow during the period indicated by the *horizontal bars* while recording the fura-2 F_{340}/F_{380} ratio used to calculate $[Ca^{2+}]_i$. Both the excitatory neurotransmitter glutamate (*Glu*, 100 μM) and the nucleotide ATP (50 μM) evoked fast and transient $[Ca^{2+}]_i$ rises. Challenging a cell with ATP (50 μM, *cell 1*) or glutamate (100 μM, *cell 2*) in the presence of the other compound did elicit a clear $[Ca^{2+}]_i$ rise over current level.

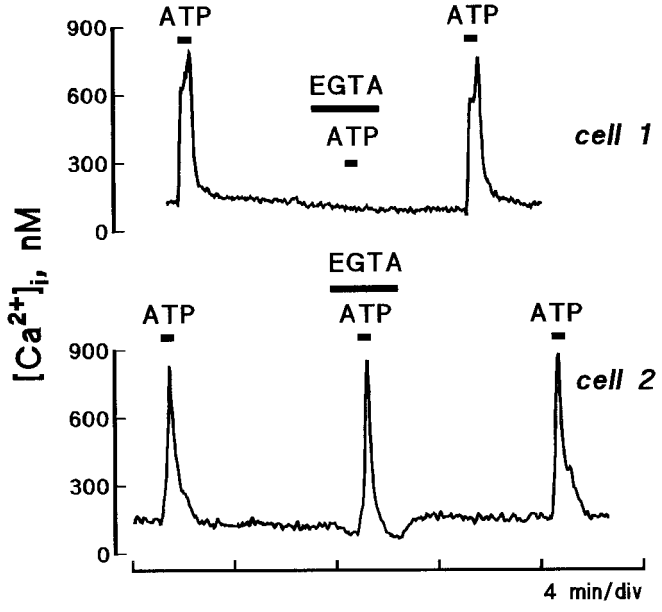

Figure 3. Extracellular Ca^{2+} dependence of ATP response in Purkinje neurons. *Cell 1,* Purkinje neuron. The cell was challenged with 50 μM ATP in normal medium (2.5 mM $[Ca^{2+}]$) before and after exposure to low Ca^{2+}. The reduction of free extracellular $[Ca^{2+}]$ to ~200 nM by perfusion with EGTA- and Ca^{2+}-buffered medium (*EGTA*) completely abolished the response to 50 μM ATP. The effect of ATP was completely recovered after reintroduction of normal medium. As a control, the response elicited by ATP in an astrocyte (*cell 2*) in the same culture was not altered by the removal of extracellular Ca^{2+}. This protocol was reproduced in 15 Purkinje cells with similar results.

ment. To verify the involvement of extracellular Ca^{2+} in $[Ca^{2+}]_i$ transients elicited by ATP, experiments were performed using Mn^{2+} as a surrogate for Ca^{2+}. Mn^{2+} binds to fura-2 and quenches its fluorescence excited at all wavelengths: F_{340}, F_{380}, and the isosbestic wavelength F_{360}. $[Ca^{2+}]_i$ can be estimated from paired ratios (i.e., F_{340}/F_{380}), whereas the F_{360} signal reports the formation of fura-2–Mn^{2+} complex. Figure 4 shows simultaneous $[Ca^{2+}]_i$ (from the fura-2 F_{340}/F_{380} ratio) and normalized F_{360} fluorescence recordings in a single Purkinje cell. In the absence of Mn^{2+}, the unmodified F_{360} trace during ATP challenge confirmed that this was indeed a Ca^{2+}-insensitive wavelength. A second ATP challenge in the presence of Mn^{2+} (in nominal Ca^{2+}-free medium) resulted in a marked drop in the F_{360} fluorescence trace, indicating the opening of an Mn^{2+}-permeable pathway in the cell membrane that allows extracellular Mn^{2+} to enter into cytosol and to bind to intracellular fura-2. Thus, the ATP receptor present in Purkinje cells should be a P2X ionotropic receptor permeable to Ca^{2+} (and Mn^{2+}) or, at least, capable of depolarizing the cells to a sufficient extent to activate voltage-dependent Ca^{2+} channels.

Contribution of voltage-activated calcium channels to ATP effect

Other ATP-gated channels have been shown to be quite permeable to Ca^{2+} ions (Benham and Tsien, 1987; Evans et al., 1996; Soto et al., 1996). To determine the relative importance of Ca^{2+} entry through the ATP receptor itself and through voltage-activated Ca^{2+} channels to ATP-elicited $[Ca^{2+}]_i$ transients, Purkinje cells were stimulated with ATP after Ca^{2+} channel blockade. However, this approach proved problematic. Even using a relatively high concentration (100 μM) of the nonselective inorganic antagonist Cd^{2+}, we could not completely block the $[Ca^{2+}]_i$ transient elicited by 30 mM K^+ depolarization in most Purkinje cells tested. Nevertheless, in those cells in which Cd^{2+} completely abolished the response to high K^+, ATP was still able to elicit a reduced $[Ca^{2+}]_i$ increase in the presence of Cd^{2+} (Fig. 5). Thus, the ATP receptor itself can allow the entry of Ca^{2+}.

Because most Ca^{2+} channels are not only voltage-activated but also voltage-inactivated, an alternative approach was followed to eliminate them; raising extracellular $[K^+]$ by 30 mM depolarizes the membrane (by ~40 mV from rest in the conditions used) and should promote inactivation of Ca^{2+} channels. Such a depolarization treatment during 10 min consistently and reliably reduced the $[Ca^{2+}]_i$ increase evoked by 60 mM K^+ to 12 ± 4% of control (Fig. 6, *bottom panel*), indicating a substantial inactivation of voltage-sensitive calcium channels. On the other hand, the signal elicited by 50 μM ATP was much less sensitive to depolarization; it was reduced only to 52 ± 7% by high K^+ pretreatment (to avoid the effect of a reduced driving force, the ATP challenge was delivered in a normal K^+ medium) (Fig. 6, *top panel*).

Effect of ATP analogs and purinoceptor antagonists

The relative potency of several analogs and antagonists was investigated to gain insight on the identity of the P2X purinoceptor mediating the $[Ca^{2+}]_i$ responses. The ATP receptor could also be activated by 2MeSATP with a similar maximal effect with respect to ATP. The ratio of peak response elicited by these two agonists was 0.93 ± 0.22 (n = 9) at 50 μM each. Other purinergic compounds, such as α,β-meATP, UTP, diadenosine polyphosphates (Ap_4A and Ap_5A), and adenosine, were essentially inactive, even when tested at 100 μM (Fig. 7). ATP was almost twice

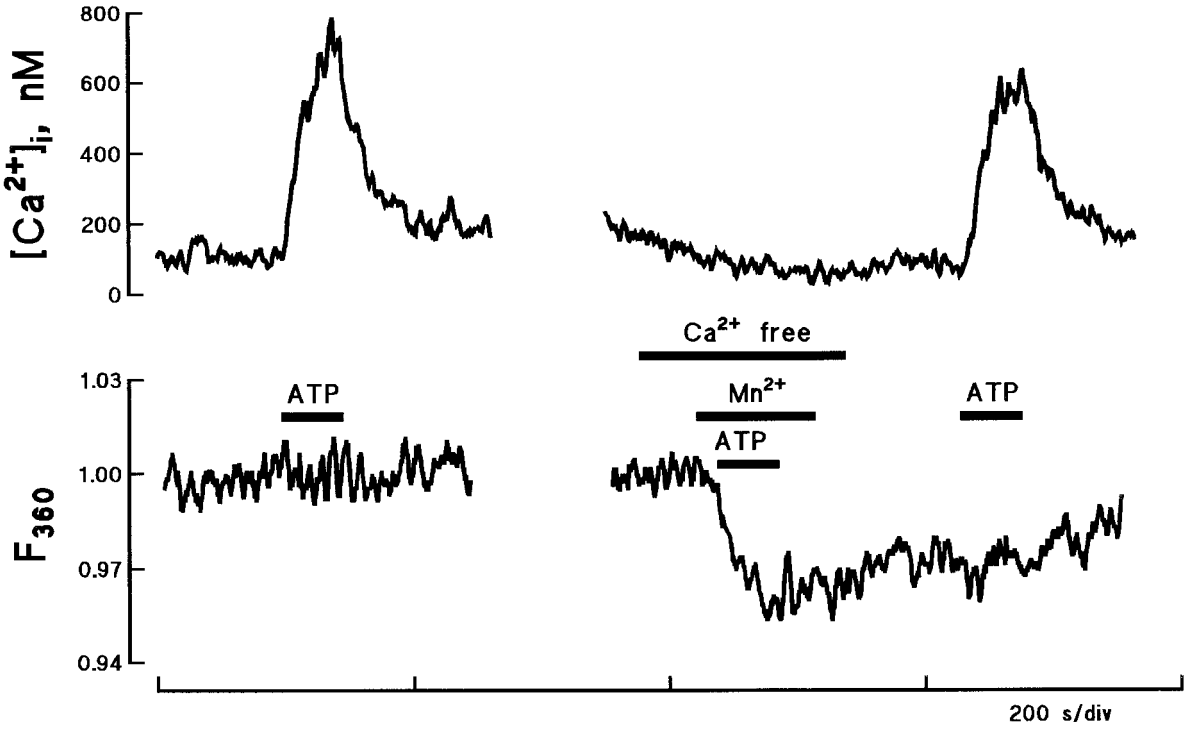

Figure 4. ATP-evoked Mn^{2+} influx in Purkinje neurons. The $[Ca^{2+}]_i$ measured from F_{340}/F_{380} ratio (*top traces*) and the normalized (F/F_0) fluorescence at 360 nm (*bottom traces*) were simultaneously recorded from single Purkinje neurons. The cell in the study was initially challenged with 50 μM ATP in normal 2.5 mM Ca^{2+} medium (*leftmost traces*). After washing out ATP and allowing a resting period of 4–5 min (*trace breaks*), a nominally Ca^{2+}-free medium (no added Ca^{2+}, no added EGTA) was introduced, followed by 200 μM Mn^{2+} in this Ca^{2+}-free medium. A subsequent challenge with 50 μM ATP in the presence of Mn^{2+} resulted in a drop in F_{360} fluorescence attributable to intracellular fura-2 quenching by entering Mn^{2+}, without an accompanying fura-2 ratio signal. After washout of Mn^{2+} and returning to normal medium, another 50 μM ATP challenge elicited a fully recovered $[Ca^{2+}]_i$ response but no F_{360} effect. The whole experiment was repeated in three cells with identical results.

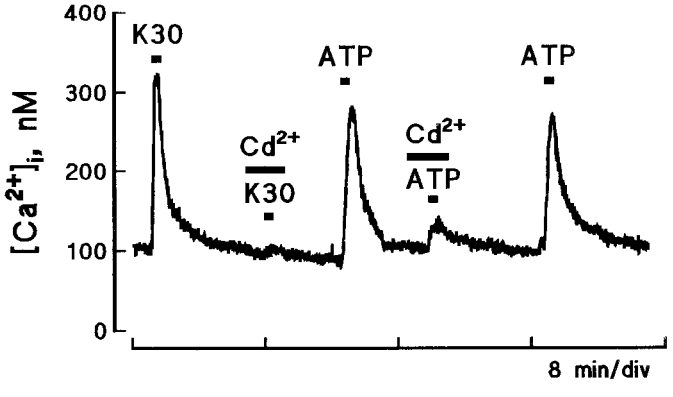

Figure 5. Effect of blockade of voltage-dependent Ca^{2+} channels by Cd^{2+}. An example of a Purkinje cell in which Cd^{2+} (50 μM) completely abolished the $[Ca^{2+}]_i$ transient because of activation of voltage-dependent Ca^{2+} channels by depolarization with 30 mM KCl (*K30*). ATP (100 μM) was still able to elicit a $[Ca^{2+}]_i$ rise in the presence of 50 μM Cd^{2+}.

as potent as 2MeSATP, with EC_{50} values of 10.6 ± 0.7 and 21.7 ± 1.9 μM, respectively (Fig. 8). The effect of ATP and 2MeSATP was dependent on concentration in a steeper way than a 1:1 binding, because apparent Hill numbers were 1.7 ± 0.2 for ATP and 1.3 ± 0.1 for 2MeSATP.

On the other hand, the $[Ca^{2+}]_i$-increasing effects of ATP and 2MeSATP were reduced by the purinoceptor antagonist suramin and PPADS (Fig. 9). The block was not complete, amounting to an 81 ± 5% (n = 17) inhibition for 100 μM suramin acting on ATP (at 50 μM) and a 77 ± 9% (n = 9) for 10 μM PPADS on 50 μM ATP. However, the antagonist effect of both compounds, which was observed in all Purkinje cells tested, was established rapidly (within 1 min) and was partially reversible after removal of the antagonist.

$[Ca^{2+}]_i$ responses in granule cells

We also tested the effect of ATP in granule cells, which constitute the vast majority of the neurons in the cultures. Figure 10 shows that, in our culture conditions, ATP failed to elicit fura-2 signals in cells that displayed healthy and characteristic responses to high KCl and to glutamate (peak followed by an increasing ramp). The protocol illustrated in Figure 10 was strictly applied in 15 cells, but >50 granule cells were challenged with ATP from day 1 to day 7 *in vitro* without positive responses. The lack of response to ATP might be attributable to receptor desensitization by extracellular ATP. We measured [ATP] in culture medium after HPLC separation (Mateo et al., 1997) and found only minor amounts, <0.2 μM, close to the detection limits in all cases. In addition, the lack of response to ATP persisted after extensive washing with ATP-free solution for >2 hr in the perifusion chamber. Times for recovery from desensitization in $P2X_1$ or $P2X_3$ receptors are typically much shorter (10–20 min; see Valera et al., 1994; Evans et al., 1995; Lewis et al., 1995).

DISCUSSION

Purkinje neuron cultures are usually established from embryonic tissue, because the appearance of this cell type occurs early in

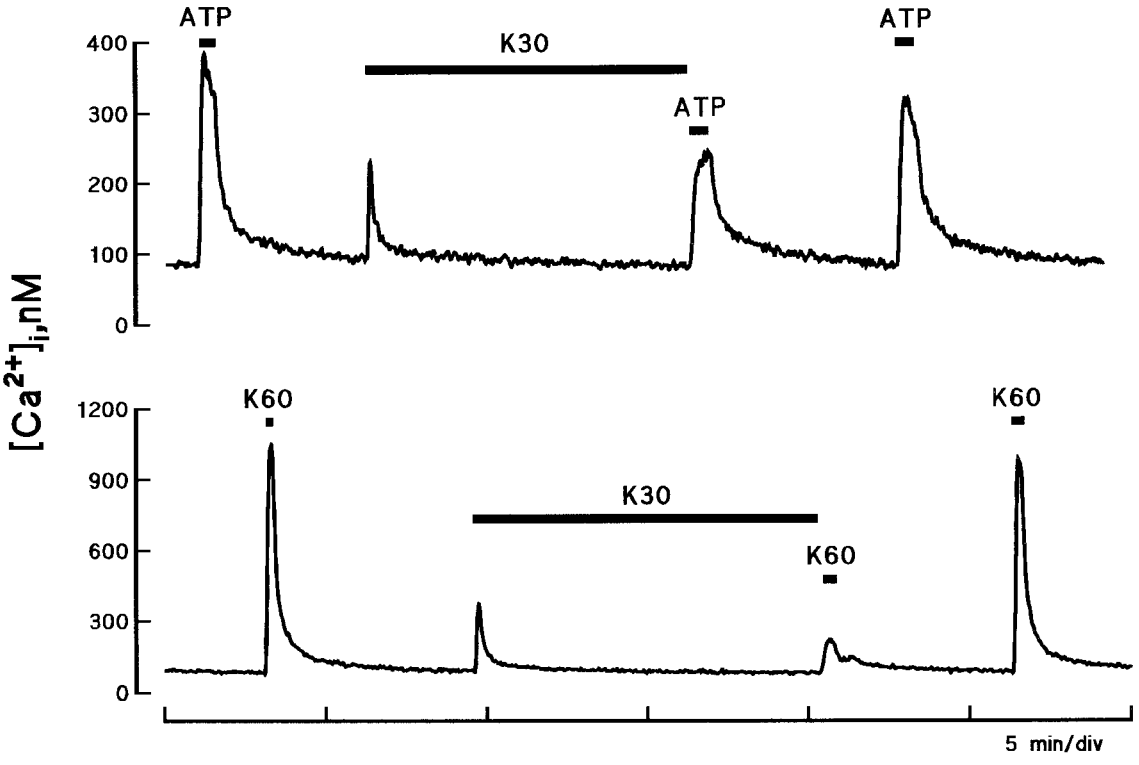

Figure 6. Contribution of voltage-dependent Ca^{2+} channels to ATP response. Purkinje neurons were depolarized for 10 min by superfusion with a 30 mM KCl solution (*K30*) to inactivate voltage-dependent Ca^{2+} channels. Exposure of cells to this solution elicited a transient increase in $[Ca^{2+}]_i$ that returned to basal levels within minutes despite the presence of high K^+, indicating inactivation of Ca^{2+} channels. Then the cells were challenged with 50 μM ATP (in normal K^+ medium, *top panel*) or 60 mM KCl-containing medium (K60, *bottom panel*). The response to test challenge after predepolarization was compared with control responses elicited by 50 μM ATP or 60 mM KCl well before (>5 min) and well after the combined test pulse protocol.

cerebellum development. However, the use of embryonic cell cultures is complicated by the developmental changes that occur during maturation of Purkinje cells. It is known that the expression of receptors for glutamate or GABA varies during development in Purkinje neurons, either in the amount of receptors (Hockberger at al., 1989) or in the subunit composition of the receptors present in Purkinje neurons (Akazawa et al., 1994; Farrant et al., 1994; Zheng et al., 1994). Therefore, we have preferred to obtain these cells at a more mature stage, which could resemble more reliably the properties of adult Purkinje cells, rather than obtaining them from embryos. Our results indicate that calbindin-immunoreactive cells can be found in cultures established from later postnatal rat cerebellum. Calbindin-immunoreactive cells are unequivocally identified as Purkinje neurons, because this protein is expressed solely by this cell type in the rat cerebellum (Garcia-Segura et al., 1984; Wood et al., 1988). The very fact that these cells do not emit neurites in culture confirms that they are in a more differentiated state than cells obtained from embryonic tissue.

To our knowledge, this is the first report of ATP-elicited fast Ca^{2+} entry signals in cerebellar Purkinje cells. The absolute dependence on extracellular Ca^{2+} implies that the receptor mediating these signals is an ionotropic one. In contrast with this conclusion, ATP-elicited $[Ca^{2+}]_i$ transients are only partially reduced in mouse Purkinje cells studied *in situ* in cerebellar slices (Kirischuk et al., 1996). However, in that study Purkinje cells were loaded with fura-2 by incubation of the whole slice with fura-2 AM; thus other cells in addition to Purkinje neurons could be loaded. Purkinje neurons *in situ* are completely ensheathed by glial cell processes (Palay and Chan-Palay, 1974), and it may be difficult to determine the origin of the fura-2 signal, whether the Purkinje cell itself or surrounding glia. This is important because glial cells do express metabotropic P2 receptors (Fig. 3) (Kirischuk et al., 1995). The use of isolated cells in culture have allowed a more definite analysis. In particular, the stimulation of Mn^{2+} entry by ATP clearly indicates that ATP opens, directly or indirectly, an ionic pathway that allows Mn^{2+} influx across the membrane. Either the ATP receptor itself is permeable to Mn^{2+} ions (and presumably to Ca^{2+}), or stimulation of ATP receptor depolarizes Purkinje neurons (via Na^+–K^+ channels) in a sufficient extent to activate voltage-dependent Ca^{2+} channels (again an ionotropic mechanism). Thus, we consider that the ATP receptor present in rat Purkinje cells is an ATP-gated channel belonging to the P2X purinoceptor family. This channel also seems to be permeable to Ca^{2+} ions, based on the ability to evoke a $[Ca^{2+}]_i$ increase after complete blockade of voltage-dependent Ca^{2+} channels by Cd^{2+}. In fact, the permeability of other ATP-gated channels to Ca^{2+} is quite high. The fraction of current actually carried by Ca^{2+} ions is higher in ATP receptors than in acetylcholine nicotinic receptors (Rogers and Dani, 1995). However, Cd^{2+} may also block the P2X channel (Nakazawa and Hess, 1993; Evans et al., 1996) and thus precludes a quantitative conclusion about how much Ca^{2+} flows through the ATP-gated channel. The voltage-dependent calcium channels do contribute to the $[Ca^{2+}]_i$ signal elicited by ATP, as indicated by the reduction of ATP-evoked transient by inactivation of calcium channels with a depolarizing prepulse. However, a 90% inactivation of calcium channels reduced ATP-evoked signal by <50%, indicat-

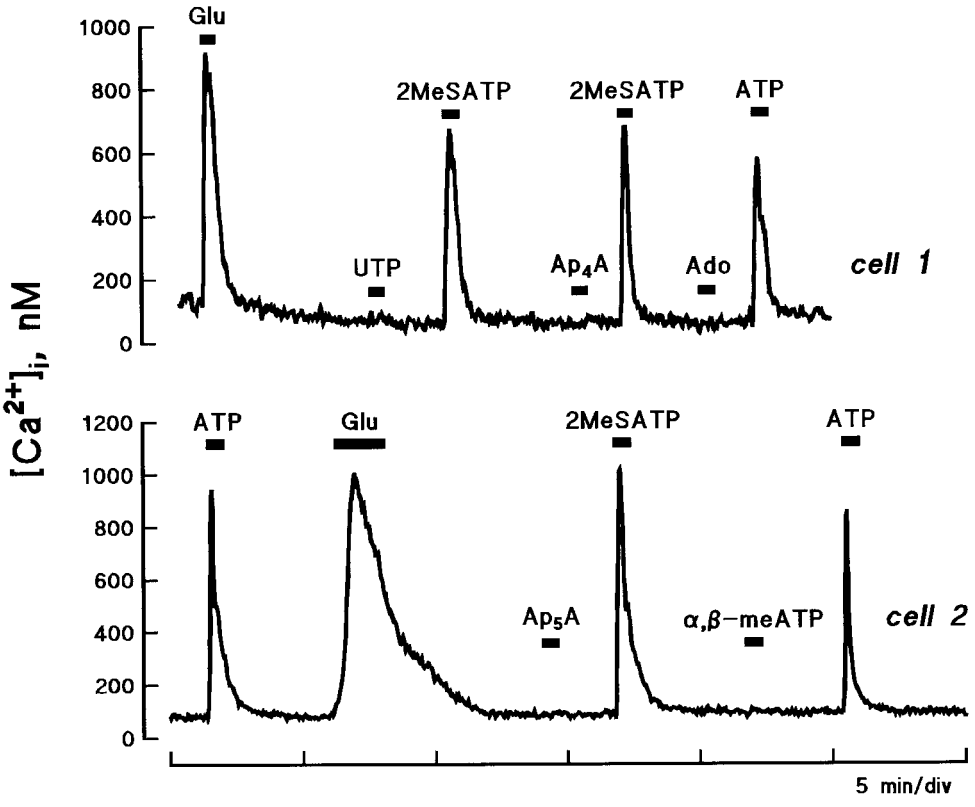

Figure 7. Responses to purinoceptor agonists in Purkinje neurons. $[Ca^{2+}]_i$ recording traces are presented for representative cells. Drugs were applied with the superfusion flow for the period indicated by the *bars*. The cells were always challenged with glutamate (100 μM); only cells showing healthy responses to glutamate were selected to test the other compounds. All Purkinje neurons tested responded to ATP with vigorous $[Ca^{2+}]_i$ increases. The responses to 2MeSATP and ATP were of similar magnitude in nine cells in which the two agonists were tested. Purkinje cells were insensitive to UTP (n = 23), the diadenosine polyphosphates Ap_4A and Ap_5A (n = 15), α,β-meATP (n = 13), and the P1 agonist adenosine (*Ado*, n = 3). All drugs were tested at 50 μM except adenosine and glutamate (*Glu*), which were used at 100 μM.

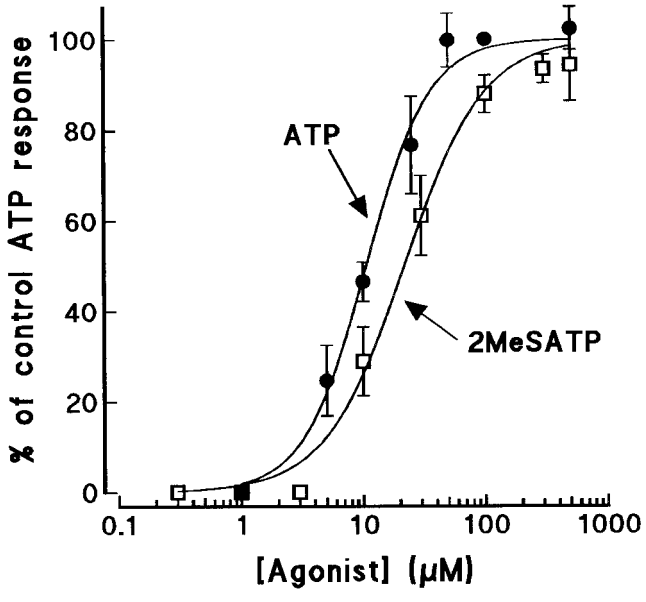

Figure 8. Concentration–response curves for purinergic agonists. Complete concentration–response curves (at least 5 points) were constructed in five individual Purkinje neurons for each ATP (*solid circles*) and 2MeSATP (*open squares*). Data were normalized with respect to the response elicited by a 100 μM ATP challenge used as control in each cell and pooled to form the combined curves shown. *Lines* are logistic curves fitted to data by a nonlinear least squares routine (Fig.P program; Biosoft).

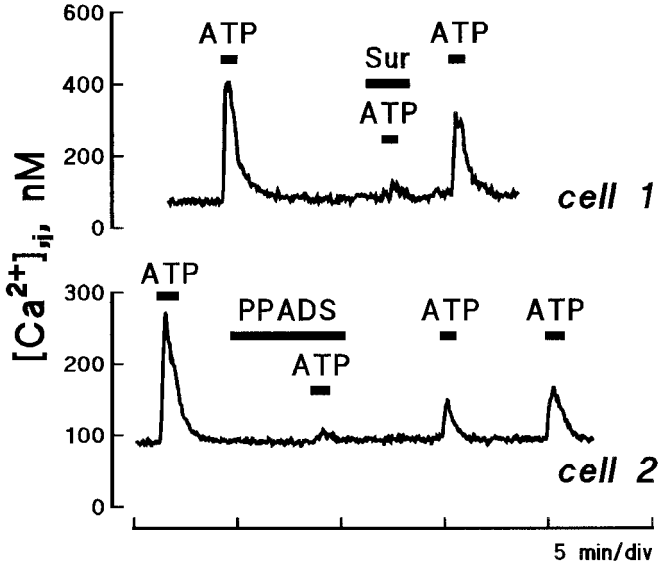

Figure 9. Effect of the purinoceptor antagonists suramin and PPADS on nucleotide-evoked $[Ca^{2+}]_i$ increases. Suramin almost completely inhibited the $[Ca^{2+}]_i$ rise elicited by ATP in Purkinje neurons (*cell 1*). Suramin was introduced 1 min before the ATP challenge. The inhibitory effect of suramin was rapidly reversible after washout. Exposure to PPADS for 1–5 min also blocked the response to ATP in a partially reversible way (*cell 2*). ATP and suramin were tested at 50 μM; PPADS was used at 10 μM.

ing that a significant portion of it is independent of calcium channels. If the relative contributions of Ca^{2+} entry through the ATP channel and the voltage-dependent channels is not altered by the prepulse, as much as 40% of the ATP-evoked $[Ca^{2+}]_i$ signal can be attributed to the operation of the ATP channel.

The pharmacology of the endogenous P2X receptor present in Purkinje cells resembles that showed by the first "neuronal" P2X receptor cloned, $P2X_2$: equipotently activated by ATP and 2MeSATP, insensitive to α,β-meATP, and blocked by suramin and PPADS (Brake et al., 1994; Evans et al., 1995). In fact, the $P2X_2$

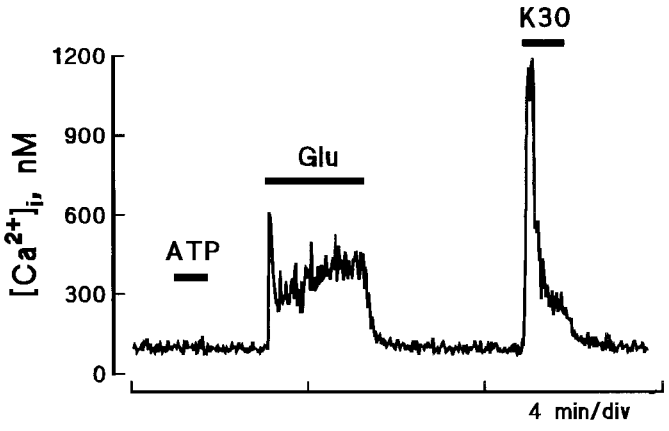

Figure 10. Response of cerebellar granule cells to depolarizing agents and ATP. Glutamate (*Glu*, 100 μM) and KCl (30 mM) were able to elicit clear $[Ca^{2+}]_i$ rises in granule cells. The shape of the glutamate response was characteristic, comprising an initial peak that returned to near basal levels followed by a steadily increasing wave. In our culture conditions ATP (100 μM) failed to elicit a fura-2 signal in any granule cell tested ($n > 50$).

subunit mRNA expression in cerebellum seems to be relatively high in neonatal [postnatal day 5 (P5)] rats, although it is much lower in adult animals (Kidd et al., 1995; Kanjhan et al., 1996). Moreover, despite an early negative report (Vulchanova et al., 1996), the $P2X_2$ protein has been detected in Purkinje neurons of adult rats (Kanjhan et al., 1996). Several other P2X purinoceptor subunits have recently been found to be expressed in the cerebellum. $P2X_4$ and $P2X_6$ are highly expressed by Purkinje cells in adult rats (Collo et al., 1996). Similarly to $P2X_2$, the expression of $P2X_1$ subunit mRNA seems to be much lower in adult animals but relatively high in neonatal, P5, rats (Kidd et al., 1995; Kanjhan et al., 1996). This variety raises the question of which subunits contribute to the endogenous P2X receptor present in Purkinje cells.

$P2X_1$ subunits are unlikely to contribute to the endogenous P2X purinoceptor, because $P2X_1$ receptors present a 10 times lower EC_{50} for ATP (Valera et al., 1994), and α,β-meATP is inactive in Purkinje cells. This finding is in direct contrast to other studies that have described very intense [^{3}H]α,β-meATP binding in cerebellar cortex (Bo and Burnstock, 1994; Balcar et al., 1995). This binding extends beyond the Purkinje cell layer, being very dense in the granule cell layer and thus suggesting that $P2X_1$-containing receptors may be expressed by granule cells rather than Purkinje neurons. Nevertheless, we have failed to record ATP-elicited $[Ca^{2+}]_i$ transients from granule cells. At present we do not know whether this lack of ATP sensitivity represents a genuine deficit of P2X receptors in these neurons or reflects a downregulation phenomenon attributable to culture conditions. ATP-driven receptor desensitization is probably not the mechanism underlying the lack of response, because extracellular [ATP] in the cultures was quite low. Furthermore, even after extensive washing (for >2 hr), the ATP (or α,β-meATP) sensitivity was not restored (in either granule or Purkinje cells). On the other hand, simple ATP-induced desensitization would not explain the disappearance of $P2X_2$-mediated responses in granule neurons but not in Purkinje cells. Astrocytes, which are abundantly present in the granule cell layer, can also express P2X purinoceptors (Magoski and Walz, 1992; Walz et al., 1994). On the other hand, [^{3}H]α,β-meATP labeling could represent non-P2X ATP binding sites: ectonucleotidases and other ATP-binding proteins.

Because homomeric $P2X_4$ and $P2X_6$ receptors expressed in *Xenopus* oocytes or human embryonic kidney cells have the unique pharmacological property of being resistant to blockade by suramin (up to 300 μM) and PPADS (up to 100 μM) (Bo et al., 1995; Buell et al., 1996b; Collo et al., 1996), it is interesting to emphasize that endogenous P2X purinoceptors found in Purkinje neurons are sensitive to suramin and PPADS blockade at much lower concentrations. There may be other differences: the potency ratio for ATP and 2MeSATP at $P2X_4$ homopolimers in heterologous expression systems is quite low in some (but not all) studies (Bo et al., 1995; Séguéla et al., 1996), indicating a low activity for 2MeSATP as agonist, whereas in our system ATP and 2MeSATP are almost equivalent in eliciting $[Ca^{2+}]_i$ rises. Thus, the overall pharmacology of ATP-elicted Ca^{2+} signals in Purkinje cells is not in agreement with the properties of $P2X_4$ and $P2X_6$ homopolymers studied in expression systems. In fact, the observed pharmacology is similar to that exhibited by receptors formed by $P2X_2$ subunits. Thus we favor the hypothesis that the properties of P2X purinoceptors in neonatal Purkinje cells are dominated by $P2X_2$ subunit expression. Therefore, a replacement of $P2X_2$ for $P2X_4$ and $P2X_6$ subunits during development of cerebellum may take place, in a similar way to known changes in $GABA_A$ or NMDA receptors (Akazawa et al., 1994; Farrant et al., 1994; Zheng et al., 1994). On the other hand, if $P2X_2$, $P2X_4$, and $P2X_6$ are co-expressed, our results suggest that $P2X_2$ subunits would govern the antagonist pharmacology, just as in the case of $P2X_2$ and $P2X_3$ co-assembly (Lewis et al., 1995).

Synaptic release of ATP has not been demonstrated in the cerebellum. However, there is a moderately dense noradrenergic innervation impinging onto Purkinje cells, in addition to other cell types in granular and molecular layers (see Palay and Chan-Palay, 1974; Wood et al., 1992). In the periphery, co-storage and co-release of ATP from noradrenaline-containing nerve terminals is well established (Stjärne, 1989). In fact, ATP is responsible for fast neuromuscular transmission to smooth muscle in organs such as vas deferens, with the amine serving a secondary, slow, role (Sneddon et al., 1982; von Kugelgen et al., 1994). Our results open the possibility of the presence of purinergic nerves in the cerebellum, suggesting the involvement of ATP in informational processing in this brain region. It should be remembered that Purkinje neurons do not express either NMDA receptors or Ca^{2+}-permeable AMPA receptors (see Eilers et al., 1996), and thus the presence of ATP receptors highly permeable to Ca^{2+} may be very relevant for synaptic plasticity at Purkinje cell inputs.

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