Purinergic Receptors and Their Activation by Endogenous Purines at Perisynaptic Glial Cells of the Frog Neuromuscular Junction

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Glial cells are closely associated with synapses and are modulated by neurotransmitters released during synaptic transmission. At many synapses, ATP is released during synaptic transmission and is involved in cell–cell signaling. Since glial cells have purinoceptors, it is possible that ATP mediates synaptic neuron–glia signaling. This work aims at determining which types of purinoceptors are present on perisynaptic Schwann cells, the perisynaptic glial cells at the frog neuromuscular junction, and test their sensitivity to endogenous purines by monitoring the relative changes of intracellular Ca^{2+} . Local application of ATP induced the release of Ca^{2+} from internal stores. Adenosine induced Ca^{2+} responses that were blocked by A1 receptor antagonists and mimicked by an A1 receptor agonist and were caused by the release of Ca^{2+} from internal stores via a pertussis toxin-sensitive G-protein. A2 receptor antagonists had no effect on Ca^{2+} responses induced by adenosine. Me-S-ATP, an ATP analog, triggered Ca^{2+} release from internal stores via a pertussis toxin-sensitive G-protein, consistent with the activation of P_{2Y} receptors. L-AMP-PCP, another ATP analog, induced Ca^{2+} entry mainly through L-type Ca^{2+} channels by a pertussis toxin-insensitive mechanism, consistent with the activation of P_{2X} receptors. Blockade of adenosine receptors did not affect glial Ca^{2+} responses induced by nerve evoked transmitter release. However, blockade of ATP receptors reduced the size and increased the delay of the responses. Hence, purinoceptors are present on the perisynaptic Schwann cells and are activated by endogenous ATP released during synaptic transmission.

[Key words: adenosine, ATP, Ca^{2+} , Ca^{2+} channels, purinoceptor, Schwann cell, glial cell, transmitter release, synapse, neuromuscular junction]

Glial cells are active components of the nervous system, possess numerous types of receptors for different neurotransmitters as well as a large variety of ion channels (Hamprecht, 1986; Barres

et al., 1990). Equipped with this cellular machinery, glial cells closely associated with synapses react to the release of neurotransmitters during synaptic transmission (Chiu and Kriegler, 1994). Synaptic activity generates Ca^{2+} responses in a network of astrocytes in the hippocampus (Dani et al., 1992) and the release of neurotransmitters by a single synapse at the frog neuromuscular junction is sufficient to induce Ca^{2+} responses in perisynaptic glial cells (Jahromi et al., 1992; Reist and Smith, 1992). These responses are mimicked by local applications of neurotransmitters present in synaptic vesicles (Jahromi et al., 1992, 1993).

In addition to ACh, a large concentration of ATP is also released during synaptic transmission (Smith, 1991; Zimmermann, 1994). More than playing its ubiquitous role in energy supply, ATP has been proposed to be involved in intercellular signaling in various systems of the peripheral and central nervous systems (Sneddon et al., 1982; Sneddon and Burnstock, 1984; Lindgren and Smith, 1987; Benham, 1989; El-Moatassim et al., 1992; Evans et al., 1992; Salter and Hicks, 1994; Zimmermann, 1994; Lyons et al., 1995). Among other mechanisms, ATP has been shown to increase intracellular Ca^{2+} (O'Connor et al., 1991) either by releasing Ca^{2+} from internal stores or by regulating Ca^{2+} entry. At the frog nmj, application of ATP on perisynaptic Schwann cells (PSC) induces the release of Ca^{2+} from internal stores (Jahromi et al., 1992). The goal of this work was to characterize further the mechanisms responsible for the Ca^{2+} responses evoked by ATP in PSCs of the frog nmj and examine the involvement of endogenous purines in the modulation of synaptic glial cells *in situ*.

There are two classes of purinoceptors based upon their selectivity to adenosine (P1 receptors) and ATP (P2 receptors). Adenosine receptors are divided into two main groups, A1 and A2 receptors, based on their ability to respectively inhibit or facilitate adenylate cyclase and also on their respective structure-activity and interactions with various drugs (Linden, 1991). ATP receptors are divided in five groups. The P_{2X} , P_{2T} , and P_{2Z} receptors are permeable to various ions including Ca^{2+} , whereas P_{2Y} and P_{2U} receptors mediate the release of Ca^{2+} from internal stores via G-binding proteins (G-protein) (Burnstock, 1990; O'Connor et al., 1991; Barnard et al., 1994). Additional characterization has been achieved at the molecular level where three different genes for P_{2Y} receptors were cloned showing a strong homology to G protein-coupled receptors (Barnard et al., 1994), two P_{2X} receptors have been cloned showing homologies with the transmitter-gated ion channel superfamily (Barnard, 1992) and many adenosine receptors have been also identified at the molecular level (Müller and Scior, 1993).

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Table 1. Purinoceptors and their regulatory mechanisms at PSCs of the frog nmj

		Normal Ringer	0 Ca ²⁺ 5 Mg ²⁺	Cd ²⁺	(+)-R- Bay K 8644	Suramin	DPCPX	CPT	PTX
P2	ATP	262 ±48	NA	NA	NA	NA	NA	NA	NA
	L-AMP-PCP	121 ±14	25 ±9	12 ±8	25 ±12	10 ±4	115 ±14	NA	126 ±24
	MeSATP	212 ±29	184 ±27	NA	NA	19 ±6	203 ±25	NA	29 ±6
	Adenosine	229 ±42	159 ±26	NA	NA	215 ±20	4 ±1	51 ±18	23 ±9
	CPA	205 ±22	NA	NA	NA	NA	NA	NA	NA
	Nerve stim.	326 ±59	NA	NA	NA	87 ±45	396 ±69	NA	NA

Values are the mean ± SEM of the relative changes of fluorescence ($\Delta F/F$) obtained following local applications of agonists in various experimental conditions. Data are grouped according to the agonist specificity for P1 or P2 receptors. Data from nerve-evoked Ca²⁺ responses are also presented (Nerve stim.). NA, data not available.

We report here that PSCs at the frog nmj possess ATP and adenosine receptors. ATP but not adenosine receptors regulate nerve-evoked Ca²⁺ responses in PSCs.

Preliminary results have appeared (Robitaille, 1994).

Materials and Methods

Experiments were performed on nmjs of cutaneous pectoris muscles of *Rana pipiens* frogs. Unless stated otherwise, the physiological solution contained (in mM): 120 NaCl, 2 KCl, 1 NaHCO₃, 1.8 CaCl₂, 5 HEPES. The pH was adjusted at 7.2.

Calcium imaging of PSCs. Nerve-muscle preparations were isolated from the frogs and pinned down in a recording chamber coated with Sylgard. The membrane permeant form of the Ca²⁺ indicator fluo 3 (fluo 3-AM, Molecular Probes) was used. The fluorescence emitted by fluo 3 increases when it is bound to Ca²⁺ (Kao et al., 1989). For loading the Ca²⁺ indicator into the PSCs, preparations were incubated for 120–150 min at 21–23°C with a solution containing 10 μ M fluo 3-AM with a final concentration of 1% dimethyl sulfoxide and 0.02% pluronic acid. Heavy metal ions that limit the response of fluo 3 to Ca²⁺ were partially chelated using tetrakis (2-pyridylmethyl) ethylenediamine (TPEN, 20 μ M; Molecular Probes) (Arslan et al., 1985; Jahromi et al., 1992).

Changes in fluorescence intensity were detected using an intensified CCD camera system (Sony camera XC-77 and a Hamamatsu intensifier, C-2487) and images were digitized by a computer using the IMAGE 1 software (Universal Imaging). A 40 $\times$ water immersion lens was used (Nikon, 0.55 NA) and an additional magnification was obtained with an optizoom (Nikon). The excitation wave length was 485 ± 20 nm and emitted fluorescence was detected through a low pass filter with cutoff at 515 nm. Surface nmjs were located using transmitted light microscopy and reliable identification of PSCs was based on their shape, size and association with the motor endplate (Georgiou et al., 1994b). Neutral density filters of at least 50% were used to minimize damage of the preparations with excess of light energy. With these precautions, PSCs at nmjs could be repeatedly observed with no signs of cell damage or bleaching of the fluorescence. The fluorescence intensity (F) was measured over the PSCs cell body and the relative changes in fluorescence intensity were expressed as

$$\Delta F/F = (F - F_{\text{rest}})/F_{\text{rest}}$$

To reduce discrepancies in Ca²⁺ responses between different preparations, care was taken to use only cells that had resting fluorescence level which allowed the use of similar gain and digital offset parameters.

Drug applications. Agonists were applied locally on PSCs using micropipettes (tip diameter, 2–3 μ m). Agonist solutions were prepared with the same solution used for perfusion so that the only difference between the agonist solution and the perfusion solution was the presence of the agonist. The tip of the pipette was positioned close to the

PSCs soma under visual control at high magnification (40 $\times$ objective) and the agonist solution was applied with a 1 sec pulse of positive pressure (5–10 psi) with a Picospritzer II (General Valve, Fairfield, NJ). For experiments where external Ca²⁺ ions were replaced by 5 mM MgCl₂, preparations were incubated for 30 min with 0 Ca²⁺-5 Mg²⁺ Ringer's before imaging. When ethylene glycol bis(β -aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) was added to the 0 Ca²⁺-5 mM MgCl₂ solution, imaging was started 10–20 min after application of the EGTA solution.

Incubation with pertussis toxin. Nerve-muscle preparations were incubated for 12–16 hr at 20–22°C in normal frog Ringer's containing 2 μ g/ml of pertussis toxin (PTX, RBI). The PTX solution was replaced with fresh toxin solution at least twice during the incubation period.

Ca²⁺ responses evoked by nerve stimulation. Nerve-muscle preparations were processed for fluo 3 loading as described above. Cholinergic receptors were blocked with α -bungarotoxin (α -BuTx) (10 μ g/ml; Molecular Probes) for 10 min to prevent muscle contractions evoked by transmitter release. α -BuTx does not affect Ca²⁺ responses of the PSCs (Jahromi et al., 1992, 1993). Motor axons were stimulated at 50 Hz for 30 sec and changes in intracellular Ca²⁺ were monitored as described above. Preparations were allowed to rest for 20 min between train of stimuli when several trains were performed on the same preparation.

Drug supplies. Drugs were obtained from Research Biochemical Inc. except for EGTA (Sigma) and suramin (RBI or Calbiochem).

Statistical analyses. Statistical comparisons were performed using an analysis of variance to avoid problems caused by repeated Student *t* tests. Data were grouped around the control sample (agonist alone in normal Ca²⁺; normal Ringer's, Table 1) and compared to the changes in fluorescence obtained with the agonist alone. Thus, all experiments involving adenosine for example, were compared at once using an Analysis of variance with the values obtained with adenosine alone. A Student paired *t* test was used when two treatments were performed on the same cell and a Student *t* test was used for comparisons between different groups (for example adenosine vs ATP).

Results

ATP induces the release of Ca²⁺ from internal stores

To test whether ATP can activate PSCs, changes in intracellular Ca²⁺ induced by local application of ATP were monitored. Figure 1 shows the level of Ca²⁺ in four PSCs at rest (A1), at the peak of the Ca²⁺ response elicited by 20 μ M ATP (A2) and after recovery (A3). Similar to our earlier report, ATP induced Ca²⁺ responses in PSCs ($\Delta F/F = 262 \pm 48\%$; two muscles, 15 cells) (Table 1) even in absence of external Ca²⁺ (data not shown, see Jahromi et al., 1992). This indicates that ATP induces the release of Ca²⁺ from internal stores.

Figure 1. Ca^{2+} responses in PSCs elicited by a local application of ATP. *A*, False color images of four PSCs (arrows) where blue indicates a low level of Ca^{2+} and red a high level. *A1* shows the PSCs before local application of ATP (20 μM); *A2* shows the cells shortly after application and *A3* after recovery following drug application. *B*, Changes of fluorescence ($\Delta F/F$) of the same four PSCs shown in *A*, before and after application (arrow) of ATP (20 μM). Each trace on the graph illustrates the time course of the Ca^{2+} response for each PSC, and the numbers indicate the respective time at which images in *A* were taken. Scale bar, 20 μm .

Figure 2. Ca^{2+} response of PSCs to local application of adenosine. *A*, False color images of a PSC (arrow) before (1), shortly after local application of adenosine (10 μM) (2), and after recovery following drug application (3). *B*, Changes of fluorescence of the same PSC shown in *A*, before and after application (arrow) of adenosine (10 μM). Numbers on the graph indicate the respective time at which images in *A* were taken. Scale bar, 20 μm .

However, ecto-enzymes present in the synaptic cleft (Lai and Wong, 1991; Salter et al., 1993; Ziganshin et al., 1993) will dephosphorylate ATP to form adenosine. Since adenosine is an active substance in many cells, and at the frog nmj in particular (Silinsky, 1975; White, 1988; Meriney and Grinnell, 1991; Salter et al., 1993), it is possible that the effects observed with ATP are due to the action of adenosine. To test for the presence of adenosine and ATP receptors, a series of experiments were per-

formed to monitor the effects on Ca^{2+} responses of various agonists and antagonists of P1 and P2 receptors.

Adenosine induces Ca^{2+} responses in PSCs

When applied locally, adenosine (10 μM) induced Ca^{2+} responses ($229 \pm 42\%$; four muscles, 22 cells) in all PSCs tested. Figure 2 shows the Ca^{2+} level in one PSC before (*A1*), shortly after local application of adenosine (*A2*), and following recovery

Figure 3. A1 adenosine receptors release Ca^{2+} from internal stores via a G-protein. **A**, Changes of fluorescence of one PSC induced by applications of adenosine ($10 \mu\text{M}$) (arrows) in presence of the A1 receptor antagonist DPCPX ($2 \mu\text{M}$). Note the absence of Ca^{2+} responses. **B**, Changes of fluorescence of two PSCs before and after local applications (arrows) of adenosine ($10 \mu\text{M}$) in presence of the A1 antagonist CPT ($2 \mu\text{M}$). After washout of the antagonist (break in the x-axis), local application of adenosine (arrow) induced Ca^{2+} responses in the same two PSCs. **C**, Changes of fluorescence of two PSCs before and after application (arrow) of CPA ($2 \mu\text{M}$), an A1 agonist. Each trace on the graphs illustrates the time course of the Ca^{2+} response for a PSC. **D**, Changes in fluorescence before and after local applications (arrows) of adenosine ($10 \mu\text{M}$) on five PSCs incubated for 14 hr with PTX ($2 \mu\text{g/ml}$). Each line on the graph illustrates the time course of the Ca^{2+} response for a PSC. Note the absence of Ca^{2+} responses in these conditions. **E**, Images of two PSCs (arrows) before (1) and after (2) local application of adenosine ($10 \mu\text{M}$) following 30 min of perfusion with 0 Ca^{2+} - 5 Mg^{2+} physiological solution. Note the increase in fluorescence intensity of the two PSCs indicating an increase in intracellular Ca^{2+} . Scale bar, $20 \mu\text{m}$.

(A3). The size of Ca^{2+} responses evoked by adenosine was not statistically different from the size of responses evoked by ATP (Student *t* test, $p > 0.05$) (Table 1). These results indicate that adenosine receptors are present on the PSCs and suggest that Ca^{2+} responses evoked by ATP could be explained by the activation of adenosine receptors.

Adenosine receptors are of the A1 subtype

To determine the type(s) of adenosine receptors, we first used antagonists of P1 receptors. The antagonists were perfused on the preparation for at least 20 min prior to local applications of adenosine. The presence of 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) ($2 \mu\text{M}$), a potent, specific A1 receptor antagonist (Martinson et al., 1987; Linden, 1991; Müller and Scior, 1993) prevented the production of Ca^{2+} responses by adenosine in all cells tested (Fig. 3A; three muscles, 17 cells; $4 \pm 1\%$). The average Ca^{2+} response amplitude in presence of DPCPX was signifi-

cantly smaller than the one obtained with adenosine in absence of the antagonist (ANOVA analysis of variance, $p < 0.05$). The lack of responses was unlikely due to nonspecific effects of DPCPX since Ca^{2+} responses were evoked by local application of the muscarinic agonist muscarine ($1 \mu\text{M}$) in presence of the A1 antagonist (data not shown).

Similar results were obtained when 8-cyclopentyl-1,3-dimethylxanthine (CPT), another A1 antagonist (Bruns, 1990), was used. Indeed, Ca^{2+} responses evoked by adenosine were greatly reduced when CPT ($10 \mu\text{M}$) was present in the perfusing solution (two muscles, 11 cells; $51 \pm 18\%$) (Fig. 3B). These responses were significantly different from the responses obtained in absence of the antagonist (ANOVA analysis of variance, $p < 0.05$). Ca^{2+} responses to local application of adenosine were restored after washout of the antagonist (Fig. 3B). At concentration of 100 nM , CPT still blocked Ca^{2+} responses induced by adenosine ($52 \pm 43\%$; three muscles, 15 cells). The use of large

concentrations of A2 specific antagonists such as CP-66,713 (50 μ M) or CGS 15943 (50 μ M) (Linden, 1991) did not block Ca^{2+} responses induced by adenosine (data not shown).

Ca^{2+} responses induced by adenosine were also mimicked by an A1 receptor agonist. The average increase in fluorescence evoked by N⁶-cyclopentyladenosine (CPA; 1 μ M), a highly specific A1 agonist, was similar to the ones obtained with adenosine (Fig. 3C; three muscles, 22 cells; $205 \pm 22\%$). This value was not statistically different from the size of the Ca^{2+} responses obtained with adenosine (ANOVA analysis of variance, $p > 0.05$). These results suggest that the adenosine receptors are of the A1 type. A summary of the effects of A1 agonists and antagonists is presented in Table 1.

Adenosine releases Ca^{2+} from internal stores via PTX-sensitive G-proteins in PSCs

A1 receptors are often coupled to G-proteins and regulate the release of Ca^{2+} from internal stores through IP_3 receptors. To test whether adenosine receptors of the PSCs are coupled to a PTX-sensitive G-protein, muscles were incubated for 12–14 hr with 2 μ g/ml of PTX, and the ability of adenosine to evoke Ca^{2+} responses was evaluated. No Ca^{2+} responses were evoked in PSCs by adenosine after PTX treatment (Fig. 3D; four muscles, 30 cells; $23 \pm 9\%$), in 95% of the cells tested. Ca^{2+} responses were significantly smaller than the ones obtained in absence of PTX (ANOVA, $p < 0.05$) (Table 1).

It is unlikely that the lack of responses was due to the inability of PSCs to generate any responses after such treatment since local application of muscarine, known to release Ca^{2+} from internal stores via a PTX-insensitive mechanism (Jahromi et al., 1993), still induced Ca^{2+} responses after PTX treatment (data not shown). In addition, the presence of PTX at the nmjs was confirmed by the inability of adenosine to reduce the amount of transmitter released after treatment with PTX (Silinsky, 1975; Silinsky et al., 1990; Jahromi et al., 1993).

We next tested whether Ca^{2+} responses mediated by A1 receptors are due to the release of Ca^{2+} from internal stores. Preparations were incubated in 0 Ca^{2+} -5 mM Mg^{2+} physiological solution for 30 min prior to adenosine applications. Ca^{2+} responses could still be evoked by adenosine (Fig. 3E; three muscles, 19 cells), indicating that the source of Ca^{2+} was from internal stores and did not require the entry of Ca^{2+} into the PSCs. The size of Ca^{2+} responses ($159 \pm 26\%$) was not significantly different from the Ca^{2+} responses obtained in presence of external Ca^{2+} (Anova analysis of variance, $p < 0.05$) (Table 1). Similar results were obtained when EGTA (1 mM) was added to the physiological solution to further reduce external Ca^{2+} concentration (data not shown). Hence, these results indicate that A1 receptors of the frog PSCs are coupled to PTX sensitive G-proteins which regulate the release of Ca^{2+} from internal stores.

Are ATP receptors (P2) present on PSCs?

Different P2 receptor agonists and antagonists were used to confirm the presence of ATP receptors on PSCs. The first attempts to test for the presence of P2 receptors on PSCs were performed using 2-methylthio-ATP (Me-S-ATP), an ATP analog which preferentially binds to P_{2Y} receptors (Cusack and Hourani, 1990; Salter and Hicks, 1993; Burnstock et al., 1994). Local applications of Me-S-ATP (20 μ M) induced Ca^{2+} responses ($212 \pm 29\%$) in all cells tested (three muscles; 31 cells). Figure 4, A and B, shows three PSCs at a nmj, before (A1) and shortly after application of Me-S-ATP (20 μ M) (A2). Similar to adenosine,

Ca^{2+} responses evoked by Me-S-ATP were not significantly different from the responses evoked by ATP (ANOVA, analysis of variance, $p > 0.05$) (Table 1). It is possible that the responses evoked by Me-S-ATP are mediated through a P_{2U} receptor since this agonist has some affinity for the P_{2U} receptor (Salter and Hicks, 1994). This is unlikely since the application of UTP, the agonist for P_{2U} receptor, did not evoke any Ca^{2+} responses in the PSCs (data not shown). However, because Me-S-ATP is hydrolyzable, it is possible that part of its effect was due to activation of adenosine receptors.

To further confirm the presence of ATP receptors on PSCs we used L-adenylyl 5'-(β , γ -methylene)diphosphate (L-AMP-PCP), an ATP analog with a higher affinity to P_{2X} receptors (Cusack and Hourani, 1990). On the contrary to Me-S-ATP, L-AMP-PCP is resistant to dephosphorylation (Hourani et al., 1986) and, hence, will prevent the formation of adenosine by the ecto-enzymes. Figure 4C illustrates the changes of fluorescence induced by a local application of L-AMP-PCP (50 μ M). On average, Ca^{2+} responses evoked by L-AMP-PCP were $121 \pm 14\%$ and all cells tested responded (6 muscles, 19 cells). Ca^{2+} responses evoked by L-AMP-PCP were significantly smaller than the responses evoked by ATP (ANOVA, $p < 0.05$) (Table 1).

Antagonists of P2 receptors block Ca^{2+} responses induced by ATP analogs

Ca^{2+} responses elicited by ATP agonists should be blocked by P2 receptor antagonists if they are due to the activation of ATP receptors and not adenosine receptors. Ca^{2+} responses could not be evoked by Me-S-ATP (20 μ M) in presence of suramin (200 μ M), a nonspecific P2 antagonist (Fig. 4D; four muscles, 22 cells; $19 \pm 6\%$). Only 50% of the cells were blocked when suramin was used at a concentration of 100 μ M. The blockade of Ca^{2+} responses by suramin was reversible and responses could be obtained after removal of the antagonist (Fig. 4D). Similarly, Ca^{2+} responses evoked by L-AMP-PCP (50 μ M) were reversibly blocked by suramin (100 μ M) (three muscles, 14 cells; $10 \pm 4\%$) (Fig. 4E, Table 1). However, adenosine (10 μ M) still induced Ca^{2+} responses in presence of suramin (215 ± 20 ; three muscles, six cells) (ANOVA, $p > 0.05$) (Table 1). Moreover, Ca^{2+} responses elicited by ATP analogs were not blocked by the A1 antagonist, DPCPX (Table 1) indicating that the adenosine receptors are not blocked by suramin. These results indicate that Ca^{2+} responses evoked by ATP and its agonists are likely due to the activation of P2 receptors.

The next step consisted in determining whether the two agonists act on the same P2 receptor or on different ones. To do so, the cellular mechanisms responsible for the production of the Ca^{2+} responses were examined. P_{2Y} receptors would be expected to regulate the release of Ca^{2+} from internal stores whereas P_{2X} receptors are expected to mediate Ca^{2+} entry into the cells either directly through the receptor or by activating voltage-dependent Ca^{2+} channels due to membrane depolarization (Bean, 1992; Zimmerman, 1994).

Me-S-ATP releases Ca^{2+} from internal stores via PTX-sensitive G-proteins in PSCs

The cellular mechanisms involved in the responses induced by Me-S-ATP were first examined. PTX was used to determine whether a PTX-sensitive G-protein was involved in the production of the Ca^{2+} responses. After incubation with PTX (2 μ g/ml), Ca^{2+} responses evoked by Me-S-ATP (20 μ M) were greatly diminished (Fig. 5A) (four muscles, 48 cells; $29 \pm 6\%$). Ca^{2+}

Figure 4. ATP agonists evoke Ca^{2+} responses in PSCs. **A**, Images of three PSCs before (1) and after (2) local application of Me-S-ATP ($20 \mu\text{M}$), an ATP analog. Scale bar, $15 \mu\text{m}$. **B**, Changes of fluorescence before and after local application (arrow) of Me-S-ATP ($20 \mu\text{M}$) of the same cells illustrated in **A**. Each line on the graph illustrates the time course of the Ca^{2+} response for a PSC and the numbers indicate the respective time at which images in **A** were taken. **C**, Changes in fluorescence in one PSC before and after local application (arrow) of L-AMP-PCP ($50 \mu\text{M}$), a nonhydrolyzable analog of ATP. **D**, Changes of fluorescence before and after local applications (arrows) of Me-S-ATP ($20 \mu\text{M}$) in presence of $200 \mu\text{M}$ suramin, a P_2 antagonist. After washout of the antagonist (break in the x-axis), Ca^{2+} responses could

responses were significantly smaller than the ones evoked in absence of PTX (ANOVA, $p < 0.05$) (Table 1). However, muscarine ($1 \mu\text{M}$) induced Ca^{2+} responses in the same cells confirming the ability of the cells to respond.

We next examined whether external Ca^{2+} was necessary for the responses induced by Me-S-ATP. In absence of external Ca^{2+} (0 Ca^{2+} - 5 Mg^{2+}), Me-S-ATP evoked Ca^{2+} responses that were similar to the ones obtained in presence of external Ca^{2+} (Fig. 5B) ($184 \pm 27\%$; three muscles, 19 cells) (ANOVA, $p > 0.05$) (Table 1). Similar results were obtained when EGTA (1 mM) was added to the physiological solution (data not shown). Hence, Me-S-ATP induced the release of Ca^{2+} from internal stores via a PTX-sensitive G-protein. This is consistent with Me-S-ATP acting on P_{2Y} receptors.

External Ca^{2+} is required for Ca^{2+} responses evoked by L-AMP-PCP

If L-AMP-PCP and Me-S-ATP act via the same receptor but with different affinity, cellular mechanisms causing the Ca^{2+} responses should be the same. However, if L-AMP-PCP activates P_{2X} receptors, one would predict that Ca^{2+} responses would require Ca^{2+} entry into the cell (Burnstock, 1990; Salter et al., 1993). Furthermore, unlike P_{2Y} receptors, P_{2X} receptors are generally not coupled to G-proteins (Barnard et al., 1994).

The involvement of PTX-sensitive G-proteins was first tested. Local applications of L-AMP-PCP ($50 \mu\text{M}$), still evoked Ca^{2+} responses after PTX treatment ($126 \pm 24\%$; 16/17 cells; Fig. 5C) (ANOVA, $p > 0.05$) (Table 1). The absence of effect is not due to PTX inefficacy since no Ca^{2+} responses were evoked by adenosine on the same preparations (data not shown).

The requirement of external Ca^{2+} for the genesis of Ca^{2+} responses was tested by replacing Ca^{2+} ions by Mg^{2+} ions. Ca^{2+} responses evoked by L-AMP-PCP ($50 \mu\text{M}$) were greatly reduced ($25 \pm 9\%$; three muscles, 20 cells) (Fig. 5D, Table 1). Upon addition of external Ca^{2+} (break in the x-axis), the ability of L-AMP-PCP ($50 \mu\text{M}$) to evoke Ca^{2+} responses was restored (Fig. 5D). The entry of Ca^{2+} was further tested by using Cd^{2+} ($20 \mu\text{M}$), an ion competing for many Ca^{2+} -binding sites (Bean, 1992). Ca^{2+} responses evoked by L-AMP-PCP in presence of Cd^{2+} were significantly reduced ($12 \pm 8\%$; three muscles, 19 cells; ANOVA, $p < 0.05$) (Table 1). These results indicate that L-AMP-PCP induced Ca^{2+} entry via a PTX-insensitive mechanism and are consistent with the activation of P_{2X} receptors.

Hence, the different cellular mechanisms regulating Ca^{2+} responses evoked by Me-S-ATP and L-AMP-PCP suggest that P_{2Y} and P_{2X} receptors are present on PSCs.

L-AMP-PCP mediates Ca^{2+} entry mainly through L-type Ca^{2+} channels

The possible involvement of Ca^{2+} channels in the entry of Ca^{2+} induced by L-AMP-PCP was next investigated. Ca^{2+} channels are present in many glial cells and in PSCs in particular (Barres et al., 1990; MacVicar et al., 1991; Jahromi et al., 1992) and could be activated by membrane depolarisation induced by ions

be induced by Me-S-ATP on the same cells. **E**, Changes of fluorescence before and after local applications (arrows) of L-AMP-PCP ($50 \mu\text{M}$) in presence of $100 \mu\text{M}$ suramin. Ca^{2+} responses could be evoked in the same cells after removal of the antagonist (break in the x-axis). Each line on the graphs illustrates the time course of the Ca^{2+} response for a PSC. Different preparation than **D**.

Figure 5. Cellular mechanisms of Ca^{2+} responses triggered by ATP agonists. *A*, Changes of fluorescence in three PSCs before and after

entering the cells through $\text{P}_{2\text{x}}$ receptors. The ability of L-AMP-PCP to evoke Ca^{2+} responses was monitored in presence of various L-type Ca^{2+} channel antagonists, the most common Ca^{2+} channels in glial cells (Barres et al., 1990; MacVicar et al., 1991). In the presence of the antagonist enantiomer of Bay K 8644, R(+)-Bay K 8644 (2–20 μM), (Ferrante et al., 1989; van Amsterdam et al., 1989; Ravens et al., 1990), Ca^{2+} responses induced by L-AMP-PCP (50 μM) were reduced by 80% (five cells, three muscles; $25 \pm 12\%$) (Table 1). This reduction was reversible upon washout of the Ca^{2+} channel antagonist. Similar results were obtained with nimodipine (2 μM) (Robitaille et al., 1995). These results indicate that Ca^{2+} entry induced by L-AMP-PCP, likely due to $\text{P}_{2\text{x}}$ receptor activation, is mainly the result of L-type Ca^{2+} channels activation due to membrane depolarization.

Modulation of Ca^{2+} responses by endogenous purines

The involvement of endogenous purines in triggering Ca^{2+} responses in PSCs during synaptic transmission was next examined. Transmitter release was evoked by repetitive stimulations of the motor nerve at 50 Hz. On average, Ca^{2+} responses were $326 \pm 59\%$ (three muscles, seven cells) (Table 1), the delay before the onset was 3.3 ± 0.5 sec and the time to reach the maximum amplitude was 19 ± 5 sec (Fig. 6*A,B*). As shown in our previous study (Jahromi et al., 1992), nerve-evoked Ca^{2+} responses show a progressive rundown during successive stimulations (Fig. 6*C*, solid circles). Because of that characteristic, the effects of different blockers on nerve-evoked Ca^{2+} responses were tested on different preparations and each preparation was only stimulated once.

The presence of the A1 antagonist DPCPX (20 μM) had no effect on any of the parameters of the Ca^{2+} responses evoked by nerve stimulations (average of $396 \pm 69\%$, delay of 4.1 ± 1.2 sec, time to peak of 17 ± 6 sec; three muscles, six cells). However, Ca^{2+} responses evoked by local applications of adenosine were blocked in these conditions, indicating that the adenosine receptors were blocked. Because A1 receptors often produce inhibitory actions (Silinsky et al., 1990; Edwards and Gibb, 1993), we hypothesized that the rundown of Ca^{2+} responses could be caused by the activation of A1 receptors. As shown in

local applications (arrows) of Me-S-ATP (20 μM) following 13 hr of incubation with PTX (2 $\mu\text{g/ml}$). Each trace on the graphs illustrates the time course of the Ca^{2+} response for a PSC. Note that no Ca^{2+} responses were evoked in these conditions. *B*, Images of one PSC (arrow) before (1) and after (2) application of Me-S-ATP (20 μM) following 30 min of perfusion with 0 Ca^{2+} -5 Mg^{2+} physiological solution. Note the increase in fluorescence intensity of the PSC indicating an increase in intracellular Ca^{2+} . Different preparation than *A*. Scale bar, 15 μm . *C*, Changes of fluorescence in two PSCs before and after local applications (arrows) of L-AMP-PCP (50 μM) following 16 hr incubation with PTX (2 $\mu\text{g/ml}$). Note the increase of fluorescence even after PTX treatment. *D*, Changes of fluorescence in two PSCs before and after local applications (arrows) of L-AMP-PCP (50 μM) following 30 min of perfusion with 0 Ca^{2+} -5 Mg^{2+} physiological solution. Ca^{2+} responses could be induced in the same cells when external Ca^{2+} was reintroduced in the perfusate (break in the x-axis). Each trace on the graphs illustrates the time course of the Ca^{2+} response for a PSC. Different preparation than *C*. *E*, Changes of fluorescence of a PSC before and after local application (arrow) of L-AMP-PCP (50 μM) following 30 min of perfusion with R(+)-Bay K 8644 (2 μM), an L-type Ca^{2+} channel antagonist. A small Ca^{2+} response was observed. After removal of the antagonist (break in the x-axis), local application of L-AMP-PCP (50 μM) on the same cell induced a larger Ca^{2+} response.

Figure 6. Endogenous ATP but not adenosine modulates nerve-evoked Ca^{2+} responses of the PSCs. **A**, Images of two PSCs before (1), during (2), and after (3) sustained transmitter release evoked by repetitive stimulations of the motor nerve (50 Hz). **B**, Graph of changes in fluorescence of the same PSCs as in **A**, before during and after repetitive motor nerve stimulation (50 Hz, bar). The numbers illustrated on the graph indicate at which time the corresponding figure in **A** was taken. **C**, Normalized Ca^{2+} fluorescence expressed as a percentage of the Ca^{2+} response evoked by the first train of stimuli. Solid circles represent the rundown of Ca^{2+} responses for control preparations (seven cells, three muscles) and the solid squares represent the rundown of Ca^{2+} responses evoked in presence of the A1 antagonist DPCPX (20 μM) (four cells, three muscles). Different muscles were used for each experiment. Note that the rundown is unaffected by the A1 antagonist. **D**, Changes in fluorescence of a PSC before during and after repetitive motor nerve stimulation (50 Hz, bar) following 30 min perfusion with suramin (200 μM), a P2 antagonist. The grey zone illustrates the mean and SE of nerve-evoked Ca^{2+} responses obtained in absence of the antagonist. Note the delay in the onset of the response (the changes of fluorescence occurred after the stimulation had stopped). This response is the second largest response observed in presence of suramin (seven cells, five muscles). Scale bar, 25 μm .

Figure 6C (solid squares; four cells, three muscles), the rate of rundown as well as the degree of attenuation was the same whether A1 receptors were blocked or not. Thus, these results suggest that adenosine receptors do not modulate directly the Ca^{2+} responses induced by nerve evoked transmitter release.

We next tested whether ATP receptors modulate nerve-evoked Ca^{2+} responses of the PSCs. Blockade of ATP receptors with suramin (200 μM) produced major changes in Ca^{2+} responses evoked by nerve stimulations (Fig. 6D). Ca^{2+} responses were reduced to $87 \pm 45\%$ (five muscles, seven cells), which is statistically different from the control value (ANOVA, $p < 0.05$) (Table 1). In addition, the delay before the onset of the responses was significantly increased to 16 ± 4 sec, but the time to reach the maximum amplitude was not significantly different (14 ± 3 sec; ANOVA, $p > 0.05$). The effect on the rundown of the Ca^{2+} responses could not be tested convincingly because the initial responses would often be too small. On four cells tested, aden-

osine (10 μM) could still evoke Ca^{2+} responses after the train of stimuli indicating that the cells were responsive and that the suramin effects are not due to blockade of adenosine receptors. These experiments indicate that the activation of ATP receptors, but not adenosine receptors, is necessary for optimal Ca^{2+} responses in the PSCs during synaptic transmission.

Discussion

The results of the present study reveal the presence of various purinoceptors at PSCs of the frog nmj and that these receptors are activated by endogenous ATP released by the nerve terminal during synaptic activity.

Different purinoceptors on the PSCs

Although the complete pharmacological characterization of the purinoceptors could not be achieved due to the presence of ectoenzymes (Smith, 1991) and the difficulty to block them all (Zi-

ganshin et al., 1993), the results of the present study support the fact that there are several types of purinoceptors in PSCs. For instance, the cellular mechanisms generating the Ca^{2+} responses vary according to the agonist used. L-AMP-PCP mediates Ca^{2+} entry whereas adenosine and Me-S-ATP induce the release of Ca^{2+} from internal stores. Moreover, the action of P1 and P2 receptor blockers is specific for their respective receptor type. This rules out possible actions of ATP agonists on adenosine receptors and strongly suggest that ATP and adenosine receptors are present in PSCs.

The characterization of P2 receptors is difficult owing to the lack of specific agonists and antagonists. However, the different cellular mechanisms producing the Ca^{2+} responses suggest that there are two types of ATP receptors on PSCs. Responses induced by Me-S-ATP correspond more to a P_{2Y} receptor as suggested by the release of Ca^{2+} from internal stores via G-protein mechanisms whereas responses induced by L-AMP-PCP correspond more to a P_{2X} receptor owing to the induction of Ca^{2+} entry directly through the receptors and L-type Ca^{2+} channels. Hence, we conclude that PSCs at the frog nmj have three types of purinoceptors which mediate Ca^{2+} responses.

Specificity of pharmacological agents

The pharmacological tools available to study ATP receptors are limited and their specificity of action is not always clear. For instance, suramin has been shown to inhibit ecto-ATPase activity (Voogd et al., 1993; Ziganshin et al., 1994) and 5'-adenylylimidodiphosphate (AMP-PNP), an ATP analog related to L-AMP-PCP, has been found to inhibit the activity of ecto-ADPase (James and Richardson, 1993). In both cases, endogenous ATP or locally applied ATP would remain in the synaptic cleft longer and, hence, show more potency activating all P2 receptors. This is unlikely in the present study since Ca^{2+} responses were completely and reversibly blocked by suramin, indicating that ATP receptors were blocked. Moreover, the mechanisms producing Ca^{2+} responses induced by L-AMP-PCP are different from the responses evoked by Me-S-ATP, suggesting a specific action at P_{2X} receptors.

The presence of ectoprotein-kinases on the surface of several cells (Ehrlich et al., 1986, 1994; Zimmermann, 1994) raises the possibility that Ca^{2+} responses induced by ATP are secondary to the action of an ectoprotein-kinase. This is unlikely because Ca^{2+} responses were obtained in absence of Mg^{2+} , a cation required for the function of the enzyme (Ehrlich et al., 1986). Moreover, Ca^{2+} responses induced by ATP analogs were blocked by suramin, which further supports the involvement of ATP receptors.

Adenosine receptor pharmacology is better characterized (Daly et al., 1985; Hourani et al., 1986; Martinson et al., 1987; Bruns, 1990; Paton and Taerum, 1990; Linden, 1991; Stiles, 1992; Müller and Scior, 1993). The present results are consistent with the adenosine receptor being of the A1 subtype since A2 antagonists had no effects on Ca^{2+} responses evoked by adenosine whereas the responses were blocked by A1 antagonists and mimicked by an A1 agonist at concentrations similar or lower than the ones used in other studies (Silinsky et al., 1990; Meriney and Grinnell, 1992; Kriegler and Chiu, 1993; Salter et al., 1993; Sanger et al., 1993; Abbracchio et al., 1994).

Implications of multiple purinoceptors at PSCs

ATP has been shown to mediate fast synaptic responses in several neuronal preparations (Edwards et al., 1992; Evans et al.,

Figure 7. Model of purinoceptor activation at PSCs of frog nmj. Diagram illustrating a portion of a nerve terminal at an active zone (AZ) surrounded by synaptic vesicles (SV). A Schwann cell process (SCP) is located at proximity of the active zone. The postsynaptic element has been omitted for clarity. ATP is coreleased with ACh during synaptic transmission (1) and can activate P_{2X} and P_{2Y} receptors (2). ATP will also be degraded to adenosine by the various ecto-enzymes (3). Activation of P_{2X} receptors by ATP will induce Ca^{2+} entry partly through the ATP receptor itself but mainly through L-type Ca^{2+} channels (4). Activation of P_{2Y} receptors by ATP will trigger the release of Ca^{2+} from internal stores through the activation of a PTX-sensitive G-protein (5,8). Activation of A1 adenosine receptors (6) will result in the release of Ca^{2+} from internal stores mediated by the activation of a PTX-sensitive G-protein (7–8).

1992; Edwards and Gibb, 1993; Salter et al., 1993; Backus et al., 1994; Chen et al., 1994; Zimmermann, 1994) and, hence, ATP released during nerve-evoked synaptic transmission may also quickly activate the PSCs. In the present study, it is shown that endogenous ATP released during synaptic transmission modulates the activity of the PSCs. This regulatory effect of ATP is probably underestimated since the activation of the muscle fibers, providing approximately 50% of the total ATP (Smith, 1991), was prevented by blocking the postsynaptic receptors.

The presence of multiple purinoceptors implies that ATP activates multiple cellular pathways and control various cellular functions. Owing to the nature of the various cellular mechanisms and the enzymatic cascade required to produce adenosine from ATP, one may propose the following scheme.

Upon release from the nerve terminal (Fig. 7, step 1), ATP may act directly on two different receptors, P_{2X} and P_{2Y} (step 2). ATP will also be dephosphorylated by the ecto-enzymes to form adenosine (step 3). It is quite likely that Ca^{2+} entry mediated by the P_{2X} receptors will occur prior to Ca^{2+} released by the activation of P_{2Y} receptors because P_{2X} receptors are directly permeable to Ca^{2+} and indirectly activate L-type Ca^{2+} channels (step 4) whereas P_{2Y} receptors act by a slow process involving Ca^{2+} release from internal stores via second messengers and G-protein activation (steps 5 and 8) (Barnard et al., 1994; Burnstock et al., 1994). Due to ATP and ADP inactivation of 5'-nucleotidase (Zimmermann, 1992, 1994) causing delay in the apparition of adenosine, the activation of adenosine receptors (step 6) would occur later, after degradation of ATP. It will also lead to the activation of a G-protein (step 7) and the release of Ca^{2+} from internal stores (step 8), provided that the stores were not depleted by the activation of P_{2Y} receptors. The activation of adenosine receptors is hypothetical since no evidence was obtained in the present study. A1 and P2 receptors likely have different regulating mechanisms since ATP but not adenosine antagonists modulated the nerve-evoked Ca^{2+} responses in PSCs. We hypothesize that the early activation of P_{2X} receptors engages a cascade of events that will be completed or terminated

by the activations of P_{2Y} or adenosine receptors or both. This possibility is supported by the observations that different purinoceptors may have opposite effects on the cellular functions (Boland et al., 1992; Abbracchio et al., 1994).

Actions of purines on glial cells

Adenosine and ATP receptors are widely distributed (Burnstock, 1990; Olsson and Pearson, 1990; Schwabe et al., 1991; Salter et al., 1993) and are present on several types of glial cells such as cultured astrocytes (Gebicker-Haerter et al., 1988; Neary et al., 1988; Meghji et al., 1989; Lai and Wong, 1991; Kastritsis et al., 1992; Salter and Hicks, 1994), oligodendrocytes (Meghji et al., 1989), glioma cells (Lin and Chuang, 1993; Chueh et al., 1994), microglial cells (Walz et al., 1994), and glial cells of the rat optic nerve (Kriegler and Chiu, 1993). Among other roles, purinoceptors control the synthesis of prostaglandin (Gebicker-Haerter et al., 1988) and thromboxane A₂ (Pearce and Murphy, 1988), and regulate protein phosphorylation and dephosphorylation (Neary et al., 1991). Lyons et al. (1995) showed that ATP-mediated Ca^{2+} responses in myelinating Schwann cells were dependent on direct contact with neurons. Although numerous other functions and roles have been attributed to the activation of purinoceptors (see Kastritsis et al., 1992, for discussion), it is difficult to determine the actions of the purinoceptors in frog PSCs because very little is known about their functions. A recent observation suggests that ATP and adenosine receptors are not involved in PSC regulation the same way muscarinic receptors are. Indeed, we recently found that muscarinic receptors, but not purinoceptors, can prevent the upregulation of glial fibrillary acidic protein in the frog PSCs induced by the interruption of synaptic activity (Georgiou et al., 1994a,b).

Possible modulation of synaptic functions

The most appealing potential roles of the purinoceptors are the ones that may affect synaptic activity or the maintenance and support of synaptic integrity. For example, it has been reported that adenosine and ATP modulate transmitter release at the frog nmj (Silinsky, 1975; Silinsky, 1980; Silinsky et al., 1990; Vizi et al., 1990; Meriney and Grinnell, 1992; Fu and Huang, 1994). In the context of the present work, and the evidence of glial modulation of nerve cell activity (Parpura et al., 1994), and the possible rapid neuron-glia signaling (Murphy et al., 1993), we postulate that the effects of purines on synaptic activity may be mediated in part by perisynaptic glial cells. Indeed, experiments demonstrating the effects of purines on synapses were not performed in a way to rule out any modulation by perisynaptic glial cells. The presence of functional purinoceptors at PSCs reinforce the idea that the actions of purines on synaptic functions could be mediated by perisynaptic glial cells. Perisynaptic glial cells in the CNS could also be activated by endogenous purines and, hence, may be involved in the ATP-mediated modulation of synaptic activity (Salter et al., 1993).

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