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Spike-independent release of ATP from *Xenopus* spinal neurons evoked by activation of glutamate receptors

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As the release of ATP from neurons has only been directly studied in a few cases, we have used patch sniffing to examine ATP release from *Xenopus* spinal neurons. ATP release was detected following intracellular current injection to evoke spikes. However, spiking was not essential as both glutamate and NMDA could evoke release of ATP in the presence of TTX. Neither acetylcholine nor high K^+ was effective at inducing ATP release in the presence of TTX. Although Cd^{2+} blocked glutamate-evoked release of ATP suggesting a dependence on Ca^{2+} entry, neither ω-conotoxin-GVIA nor nifedipine prevented ATP release. N-type and L-type channels are thus not essential for glutamate-evoked ATP release. That glutamate receptors can elicit release in the absence of spiking suggests a close physical relationship between these receptors, the Ca^{2+} channels and release sites. As the dependence of ATP release on the influx of Ca^{2+} through Ca^{2+} channel subtypes differs from that of synaptic transmitter release, ATP may be released from sites that are distinct from those of the principal transmitter. In addition to its role as a fast transmitter, ATP may thus be released as a consequence of the activation of excitatory glutamatergic synapses and act to signal information about activity patterns in the nervous system.

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The components for purinergic signalling, receptors for ATP and adenosine (Ralevic & Burnstock, 1998), ecto-nucleotidases (Zimmermann, 1996) and equilibrative transporters (Anderson *et al.* 1999*a*,*b*) occur throughout the brain suggesting universal roles in neural function. Despite this, rather few examples of ATP acting as a fast transmitter, either alone or as a cotransmitter, have been described (Evans *et al.* 1992; Edwards *et al.* 1992; Bardoni *et al.* 1997; Nieber *et al.* 1997; Pankratov *et al.* 1998; Jo & Schlichter, 1999; Mori *et al.* 2001; reviewed by Robertson *et al.* 2001). This disparity between the widespread distribution of purinergic signalling components and the paucity of examples of ATP acting as a transmitter in a manner akin to glutamate or acetylcholine suggests that ATP may perform additional signalling functions.

In the *Xenopus* embryo ATP and adenosine control the temporal evolution of spinal motor patterns (Dale & Gilday, 1996). Release of ATP during motor activity increases excitability by inhibiting voltage-gated K^+ currents. Through the actions of ectonucleotidases, ATP is broken down to adenosine, which inhibits the voltage-gated Ca^{2+} currents and thus decreases excitability in the motor circuits. Eventually the effect of adenosine outweighs that of ATP and activity terminates (Dale, 1998; Brown & Dale, 2000). Although the slow accumulation of adenosine during motor activity has been studied directly (Dale, 1998), the release of ATP has not been examined. Thus the mechanisms underlying its release and the identity of the neurons that release it remain unknown.

This work describes our use of the patch-sniffing technique (Hume *et al.* 1983; Young & Poo, 1983) to directly study the release of ATP, establishing which cells release ATP and the conditions under which release occurs. Patch sniffing has been used to document fast ATP release at the neuromuscular junction (Silinsky & Redman, 1996). As in these studies which show spike-mediated release of ATP as a co-transmitter, we have demonstrated ATP release from spinal pattern generator neurons in response to stimulation by current injection, resulting in action potentials. However, we find that glutamate application is effective at evoking ATP release even in the presence of TTX to block action potentials. This suggests a very close relationship between glutamate receptors and the release of ATP.

METHODS

Neurons were acutely isolated from the stage 37/38 *Xenopus* embryo (Nieuwkoop & Faber, 1956) spinal cord according to previously described methods (Dale, 1991; Brown & Dale, 2000) and in accordance with the Animals (Scientific Procedures) Act (1986). Embryos were anaesthetized in MS222 (1 mg ml^{-1}; Sigma). The neurons were used from 30 min to 4 h after dissociation. At these times the recorded neurons possess one or more short neurites (5–30 μm long) which are the remains of the native dendrites and possibly the proximal axon.

Whole spinal cords were dissected free from *Xenopus* embryos and placed onto poly-D-lysine-coated dishes where they stuck sufficiently well to enable patch sniffing. HEK cells expressing $P2X_2$ receptors were maintained by standard procedures and plated onto glass coverslips. The coverslips with HEK cells were placed in the dish with the cord/neurons shortly before experiments began. Pipette solutions for both neurons and HEK cells contained (mM): 100 $KMeSO_3$, 5 KCl, 6 $MgCl_2$, 20 Hepes, 5 ATP, 2 BAPTA, pH 7.4. Sniffer electrodes (resistance 4–6 MΩ) were held at –50 mV in order to induce inward current in response to any channel gating. Once a whole cell recording from the HEK cell had been achieved, the pipette was drawn away from the cell. Sometimes the whole cell came away from the substrate, in which case the whole cell was used as a sniffer (sniffer cell). On other occasions an outside-out patch was formed which was used to detect ATP (sniffer patch). We have used this terminology in the figure legends to indicate those occasions when a sniffer cell or sniffer patch was used for recording.

The external recording solution contained (mM): 115 NaCl, 2.4 $NaHCO_3$, 3 KCl, 10 $CaCl_2$, 10 Hepes, pH 7.4. Mg^{2+} was omitted from the medium to prevent any blockade of the NMDA receptor and thus increase the chances of seeing ATP release during agonist applications. The neurotransmitters, cadmium and TTX were applied using a multi-barrel perfusion system in an identical solution adjusted to pH 7.0 in order to increase the sensitivity of the $P2X_2$ receptor to ATP (King *et al.* 1996). ATP solutions were applied from a separate multi-barrel perfusion system to avoid any possibility of cross-contamination of solutions. In experiments performed using the whole cord, TTX, nifedipine and ω-conotoxin-GVIA (ω-CgTx) were also added to the standard external solution (when required) in order to ensure the whole tissue was well perfused.

Patch recordings were made with an Axopatch 200A amplifier (Axon Instruments, Union City, CA, USA). Data were acquired to disk at a sample frequency of 50 kHz and filtered with a cutoff of 5 kHz and subjected to further digital filtering (cutoff 2 kHz) offline.

The sniffer currents were recognized according to the following criteria: (1) they followed the stimulus, electrical or agonist application, at an appropriate latency (up to a few hundred milliseconds following spikes, or a few seconds following agonist application); (2) they were larger in amplitude than any ongoing spontaneous gating; and (3) they exhibited an increase in current noise over the baseline (as a result of $P2X_2$ channel gating). The amplitude of the sniffer currents was measured as the peak of the response relative to the immediately preceding baseline. The duration of the sniffer currents was measured from the start of the response to the time that the response returned to baseline. For those currents that consisted of a burst of channel openings, the final closing in the burst was taken as the end of the response. For the experiments where electrical stimulation was used to evoke spikes, latency was measured from the beginning of the spike train to the beginning of the response. For agonist-evoked ATP release, a manual marker for the onset of agonist application was recorded during the data acquisition that was accurate to the nearest 500 ms. The latency was measured from this marker to the start of the response.

Statistical comparisons of frequency of occurrence were made with the *G* test (Sokal & Rohlf, 1995) to give the χ^2 values reported in the text.

RESULTS

Selectivity of sniffer membrane

HEK cells expressing $P2X_2$ receptors provided a source of membrane sensitive to ATP for patch-sniffing experiments (Hume *et al.* 1983; Young & Poo, 1983). The sniffer electrodes consisted of either a whole cell withdrawn from the substrate or an excised outside-out membrane patch. As the stability of the recording is crucial to detect small currents, only those sniffer electrodes that had a stable baseline current were used. If any recording became unstable the experiment was abandoned and the data excluded from analysis.

To examine the selectivity of our sniffer electrodes, we tested the HEK cells against a variety of agonists and transmitters active at receptors known to be present in the *Xenopus* embryo spinal cord. We found that the HEK cells responded only to ATP. Current was not elicited by glutamate, NMDA, GABA, glycine or acetylcholine. The sniffer electrodes utilizing membrane from these cells are thus highly selective ATP sensors (Fig. 1*A*). Using these electrodes we could reliably detect applied ATP at concentrations down to 10 μM. The saturation concentration was around 500 μM. The maximum current induced in a sniffer patch was around 100 pA. Any variation was presumably due to varying receptor densities. Sniffer cells were more variable (maximum response sometimes over 1 nA), due to differing cell sizes and density of receptor expression, but any with a current of less than 100 pA were discarded. Given the variability of intrinsic sensitivity for cells and patches, and the difficulty of placing the sniffer at exactly the same distance from the target cell or tissue, we have not attempted to quantify or calibrate the responses of the sniffer electrodes in terms of ATP concentration. Our use of this technique is purely qualitative to detect release or otherwise.

Release from isolated neurons

When sniffing from acutely isolated neurons we concentrated on the possible release of ATP from neural processes. The vast majority of these processes are likely to be the remains of the short dendrites that these neurons possess rather than true axons, which are much finer in diameter and less likely to be preserved (Dale, 1991). The tip of the sniffer electrode was moved to within 4–10 μm of the end of a neurite while a second patch electrode in the whole cell mode was used to inject current into the soma and monitor the resulting depolarization. In five neurons, a transient and noisy inward current was recorded by the sniffer electrode following spikes in the *Xenopus* neuron (Fig. 1*B*). This current presumably reflects gating of the $P2X_2$ channels elicited by release of ATP from the *Xenopus* neuron.

Release was more easily detected following a train of action potentials (3/12 cells) rather than single spikes (2/34 cells).

This was presumably due to a greater quantity of ATP being released. Overall the sniffer currents following spikes had a mean latency of 108 ± 17 ms, a mean amplitude of 4.6 ± 2 pA and a mean duration of 70.4 ± 20 ms. Sub-threshold current injection never caused release, presumably because soma depolarization could not sufficiently affect the release site. It was rarely possible to induce ATP release from the same cell on more than one occasion, even after a recovery period of up to 15 min. This is probably because the release of an observable amount of ATP by this method may require a large depletion in the releasable reserves.

Glutamate is the key excitatory transmitter in the generation of spinal motor patterns and most types of spinal neuron possess glutamate receptors, including those of NMDA subtype (Roberts *et al.* 1998). To test whether glutamate receptor activation might be effective at evoking ATP release, neurons were stimulated by brief application (3–7 s) of either 1 mM glutamate or 200 μM NMDA. Out of the 53 cells that were stimulated with glutamate, 8 neurons evoked transient and noisy inward currents in the sniffer electrode. Twenty-five out of the 53 cells were also stimulated with NMDA and two of these evoked responses in the sniffer electrode (Fig. 1*C*, Table 1). There was no significant difference between the proportion of cells releasing ATP in response to glutamate or NMDA. It was never possible to evoke release of ATP more than once by this method.

We found that more than one morphological type of neuron could release ATP. Of the 22 neurons from which release was observed, 2 had a monopolar cell body that merged into a large process (commissural-like morphology), while 18 were monopolar, but not commissural-like (they did not possess a large initial process), while the remainder were bipolar. On average around 90 % of commissural-

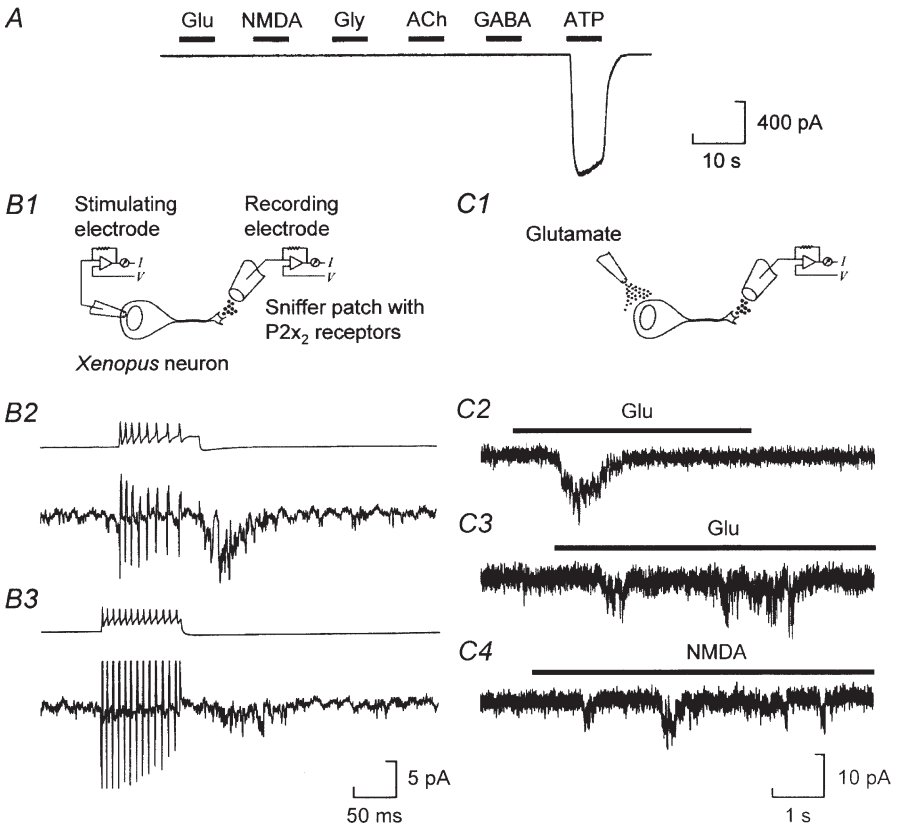

Figure 1. Patch sniffing can detect transient ATP release from isolated *Xenopus* neurons

A, whole cell recording from a HEK cell expressing $P2X_2$ receptors showing that application of 500 μM ATP resulted in a large inward current. Other transmitters and agonists (all at 200 μM) did not evoke currents. *B*1, schematic diagram to show patch-sniffing arrangement for spike-induced ATP release. *B*2, simultaneous recording from *Xenopus* neuron (upper trace) and sniffer patch showing that a train of action potentials in the neuron evokes a current in the sniffer patch (lower trace). The artifacts in the sniffer patch are field potentials evoked in the recording from the spikes in the neuron. *B*3, similar records from a different cell and sniffer patch. *C*1, schematic diagram to show patch-sniffing arrangement for glutamate-induced ATP release. *C*2 and *C*3, currents evoked in a sniffer patch (*C*2) and a sniffer cell (*C*3) by application of 1 mM glutamate (bars) to two different *Xenopus* neurons. *C*4, 200 μM NMDA could also induce ATP release (different neuron, sniffer cell).

Table 1. Characteristics of glutamate receptor-evoked ATP release from isolated neurons and spinal cords

		Frequency of ATP detection	Latency (s)	Amplitude (pA)	Duration (ms)
Isolated cells	Control	10/53 (19 %)	1.3 ± 0.3	6.7 ± 1.0	542 ± 120
	TTX	7/33 (21 %)	0.9 ± 0.2	3.2 ± 1.1*	741 ± 174
Whole cord	Control	12/28 (43 %) †	1.9 ± 0.3	15.6 ± 4.1	1696 ± 545
	TTX	8/30 (27 %)	1.2 ± 0.3	11.1 ± 4.6 ‡	1644 ± 317

The latency, amplitude and duration of currents measured by sniffer patches (mean ± S.E.M.) have been calculated from the first response to either glutamate or NMDA. † Out of 28 cords tested 8 responded to glutamate, 3 to NMDA and one to both, hence a total of 12 cords responded. ‡ As one response saturated the recording equipment, it was excluded from the calculation of the mean amplitude which has $n = 7$. This response was greater than 125 pA. * $P \leqslant 0.05$ compared to control, 2-sample t test.

like neurons and around 70 % of monopolar neurons are glycinergic (Dale, 1991), making it very likely that the glycinergic interneurons that mediate reciprocal inhibition (Dale, 1985; Dale *et al.* 1986) also release ATP as a co-transmitter. The remaining examples must represent other types of neuron that participate in motor pattern generation (Dale, 1991).

Release from isolated spinal cords

As glutamate could be used to evoke release of ATP, we investigated whether ATP release could be induced from the intact isolated spinal cord by brief application of 1 mM glutamate or 200 μM NMDA. Release was detected from 9/28 cords stimulated with glutamate and 4/18 stimulated with NMDA (Fig. 2). As with the isolated neurons there was no significant difference in the proportion of spinal cords releasing ATP in response to glutamate or NMDA. The ATP-gated currents in the sniffer electrodes were still transient but significantly larger in amplitude and duration than those recorded from dissociated cells (Table 1). The longer latency of the responses observed in response to agonist application compared to those following spikes is likely to result from perfusion delays inherent in the application system. Unlike experiments with isolated cells, it was possible in some cases to observe ATP release on more than one occasion from the same cord.

ATP antagonists block sniffer signal

To confirm that the currents observed in the sniffer patch were indeed evoked by ATP, we tested whether the P2

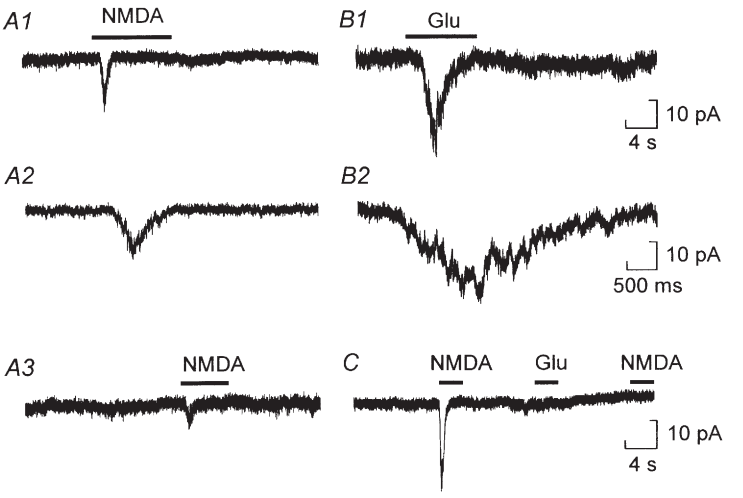

Figure 2. Glutamate or NMDA can stimulate ATP release from isolated spinal cords

*A*1, currents in a sniffer cell evoked by ATP release resulting from application of 200 μM NMDA (bar). *A*2, magnified portion of part of the same trace to show inward current has increased noise characteristic of channel activity. *A*3, currents from the same sniffer cell and spinal cord, showing that a smaller amount of ATP release could be detected after a recovery period of 4 min. *B*1, similar recording from a different experiment with a different sniffer cell, showing that 1 mM glutamate also evoked ATP release from isolated spinal cord. *B*2, part of the same record on a different time scale illustrating that the period of inward current shows increased noise characteristic of channel activity. *C*, sniffer cell current record from a separate experiment illustrating ATP detection on the first application of NMDA but no further release after re-application of glutamate or NMDA.

receptor antagonist PPADS could block the sniffer currents evoked by application of glutamate to isolated cords. Glutamate was never observed to cause a current in the sniffer patch in the presence of 20 μM PPADS ($n = 18$). In 5 out of the 18 cords tested glutamate was able to evoke small sniffer currents once the PPADS had been washed off (Fig. 3), while in the remainder sniffer currents were not observed either in the presence or absence of PPADS. The sniffer currents were thus due to release of ATP and not some other unidentified transmitter.

ATP release occurs in the absence of spiking

We next tested whether spiking was necessary for the release of ATP induced by activation of glutamate receptors. We found that even in the presence of 500 nM TTX to block action potential generation, glutamate and NMDA were still able to evoke ATP release from both isolated neurons and spinal cords (Fig. 4). TTX had no significant effect on the probability of observing ATP-release or the amplitude of the sniffer currents from isolated whole spinal cords (Table 1). However the magnitude of ATP release from isolated neurons was reduced by TTX (Table 1) suggesting that spiking during glutamate applications in the control enhanced ATP release.

Glutamate receptors selectively cause release of ATP

Activation of glutamate receptors might cause release simply by depolarizing the cells and causing a Ca^{2+} influx. In this case any excitatory stimulus might also cause spike-independent ATP release. We tested whether this might be the case with brief applications of high K^+ and the acetylcholine which will activate central nicotinic receptors on *Xenopus* neurons (Perrins & Roberts, 1995) in the presence of TTX.

High K^+, although often causing a change in the holding current of the sniffer cells (due to change in transmembrane driving force on K^+), never evoked sniffer currents in 19 cords that were tested (Fig. 5). Uniform depolarization of the spinal neuron is thus not sufficient to evoke ATP release when spiking is blocked. Similarly, brief applications of acetylcholine evoked sniffer currents in only 1/20 cords tested. By comparison glutamate evoked sniffer currents in 8/30 cords tested. These frequencies of occurrence are not

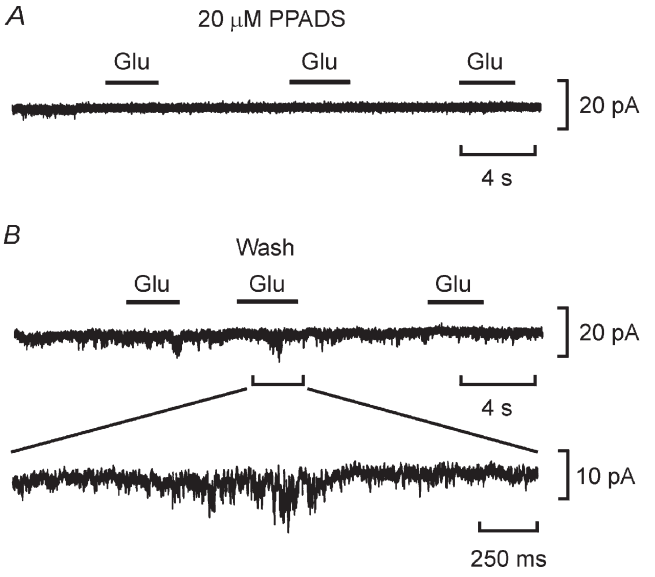

Figure 3. PPADS blocks the sniffer currents

A, in the presence of PPADS, 3 applications of glutamate to an isolated spinal cord failed to evoke a response in the nearby sniffer cell. *B*, after washout of PPADS, sniffer responses were recorded in response to 2 of 3 applications of glutamate. Lower trace shows an expanded region from the trace above indicated by the bar to illustrate ATP release evoked by glutamate. Note the large amplitude fluctuations indicative of channel gating and the increase of baseline noise in the absence of PPADS suggesting some spontaneous release of ATP from the spinal cord.

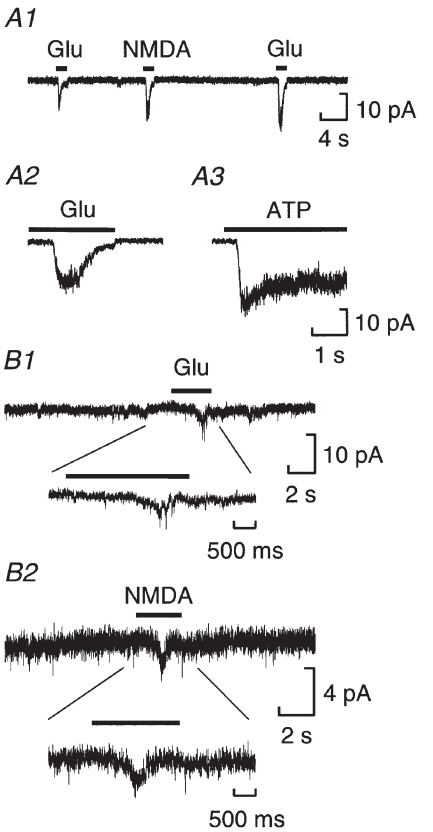

Figure 4. Spikes are not necessary for glutamate receptor-evoked ATP release

*A*1, the sniffer patch detects ATP release on application of 1 mM glutamate and 200 μM NMDA to a cord in the presence of TTX (500 nM). *A*2, part of the same record on a different time scale illustrating that the period of inward current shows increased noise characteristic of channel activity. *A*3, exogenous ATP (500 μM) evoked very similar currents in the same sniffer patch to those recorded during stimulation of endogenous release by glutamate (*A*2). *B*1, ATP release from an isolated neuron in the presence of TTX could still be detected following application of 1 mM glutamate (recorded by a sniffer patch). Inset shows an expanded portion of the trace to illustrate the sniffer currents following the application of glutamate. *B*2, NMDA application was also able to evoke release of ATP from neurons in the presence of TTX.

homogeneous ($\chi^2 = 10.7$, $P < 0.01$). Furthermore, in spinal cords where neither acetylcholine nor high K^+ evoked ATP release, glutamate could ($n = 3$, Fig. 5). We therefore conclude that activation of glutamate receptors and not other forms of excitation selectively evokes the release of ATP in the presence of TTX.

Release of ATP depends upon Ca^{2+} influx

To test the possible dependence of ATP release on Ca^{2+} influx we repeated our observations in the presence of 200 μM Cd^{2+}. Control experiments indicated that this concentration of Cd^{2+} had no effect on the currents induced by exogenous application of ATP recorded in the sniffer electrode (Fig. 6*A*). In recordings from 60 isolated cells, no inward current could be induced in the sniffer electrodes by glutamate application in the presence of Cd^{2+}, strongly suggesting that ATP release is dependent upon Ca^{2+} entry. In one cell it was possible to wash the Cd^{2+} off and subsequently detect release of ATP in response to glutamate application. For isolated spinal cords, release of ATP was seen in the presence of Cd^{2+} in only 1 out of 29 experiments ($\chi^2 = 6.9$ compared to control, $P < 0.05$). In addition to the observation that the proportion of cords releasing ATP in the presence of Cd^{2+} was statistically lower than the control, we were able to demonstrate directly block of ATP release by Cd^{2+} in 5/29 experiments. In these recordings no ATP release was seen in the presence of Cd^{2+} but was observed after the Cd^{2+} had been washed off (Fig. 6*B*). We therefore conclude that Cd^{2+} blocks the release of ATP. The entry of Ca^{2+} is likely to be through high voltage activated (HVA) channels, rather than glutamate receptors as only the former will be blocked by this dose of Cd^{2+}.

To identify the HVA channels involved, we tested whether ω-CgTx and nifedipine might block ATP release from isolated spinal cords. In neurons of the *Xenopus* embryo, about 60 % of the HVA current can be blocked by ω-CgTx (Wall & Dale, 1994), and around 8 % by nifedipine (Brown & Dale, 2000). The remainder of the current is resistant to blockade by any of the known selective HVA channel blockers.

Perhaps unsurprisingly, given its small contribution to the total HVA current, 20 μM nifedipine did not block

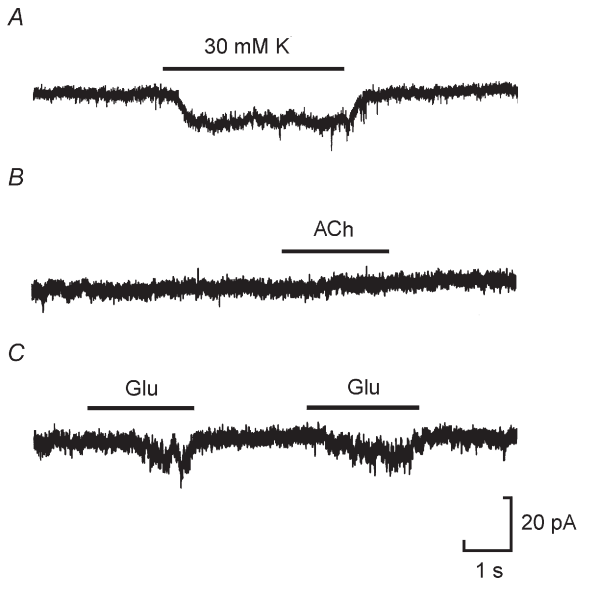

Figure 5. Glutamate but not high K^+ or acetylcholine causes release of ATP from isolated spinal cord in the presence of TTX

A, a high K^+ saline failed to evoke sniffer currents from the isolated spinal cord. *B*, application of 100 μM acetylcholine (ACh) to the same piece of spinal cord did not evoke a response in the sniffer cell either. *C*, two subsequent applications of glutamate to the same spinal cord evoked sniffer currents. The same sniffer cell was used for *A*, *B* and *C*.

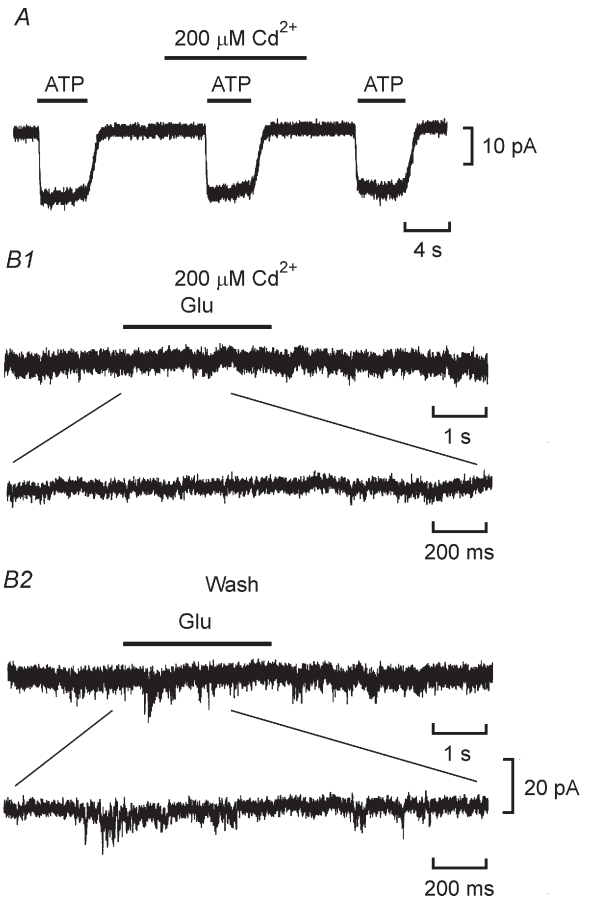

Figure 6. ATP release requires a Ca^{2+} influx

A, repeated responses to 500 μM ATP in a sniffer cell were unaffected by 200 μM Cd^{2+}, demonstrating that Cd^{2+} does not affect the sensitivity of ATP detection. *B*1, current record (sniffer cell) showing that glutamate cannot evoke release of ATP in the presence of 200 μM Cd^{2+}. Lower trace is an expanded portion of the above record during glutamate application. *B*2, after washout of Cd^{2+}, a response in the sniffer cell was seen in response to glutamate application indicating that ATP release had been restored. Lower trace is an expanded portion of the above record during glutamate application.

glutamate-induced ATP release (release seen in 4/20 cords, $\chi^2 = 0.3$ compared to control). The amplitude of the recorded currents was not significantly different from the control (Table 2, Fig. 7). We found that ATP release also persisted in the presence of 1 μM ω-CgTx (which blocks about 70 % of synaptic transmission). The frequency of release was lower than, but not significantly different from, the control (3/24 cords, $\chi^2 = 1.7$ compared to control). Furthermore when ATP release was observed in the presence of ω-CgTx the amplitude of the sniffer currents was not significantly different from the control (Table 2, Fig. 7). We conclude that although ATP release depends upon Ca^{2+} entry through HVA channels, blockade of only one subtype of channel is unable to prevent this release.

Table 2. Effect of Ca^{2+} channel blockers on glutamate-evoked ATP release from spinal cords

	Frequency of ATP detection	Mean peak amplitude (pA)
Control	8/30 (27 %)	11.1 ± 4.6 ($n = 7$)
Nifedipine	4/20 (20 %)	11.0 ± 3.7 ($n = 4$)
ω-CgTx	3/24 (13 %)	17.7 ± 10.2 ($n = 3$)
Cd^{2+}	1/29 (3 %)	—

The frequencies of occurrence of ATP release are not homogeneous ($\chi^2 = 7.4, P < 0.06$).

DISCUSSION

Mechanisms of release

We have demonstrated that *Xenopus* spinal neurons can release ATP in apparently two ways. The first involves a spike-mediated mechanism that induces release, presumably by evoking a Ca^{2+} influx at the sites of ATP release. These results are in concordance with the model of ATP acting as either a co- or principal transmitter (Evans *et al.* 1992; Edwards *et al.* 1992; Silinsky & Redman, 1996; Bardoni *et al.* 1997; Jo & Schlichter, 1999). ATP has now been identified as a co-transmitter with acetylcholine (Silinsky & Redman, 1996), GABA (Jo & Schlichter, 1999) and noradrenaline (norepinephrine) (Nieber *et al.* 1997; von Kugelgen *et al.* 1998). We have now presented evidence that ATP can be released from glycinergic neurons. However, other non-glycinergic spinal neurons will release ATP and many of these are likely to use glutamate as their principal transmitter. Our finding that trains of spikes were more effective at releasing detectable amounts of ATP are consistent with reports from other studies that have used the patch-sniffing technique (Hume *et al.* 1983). Rather than suggesting a novel mechanism of release this probably reflects the fact that the diffusion distances from the release site to sniffer are much greater than those that would be found at the synaptic cleft (4–10 μm *versus* 50 nm, respectively). Thus far greater quantities of ATP would have to be released to give a signal detectable by the sniffer electrode. Clearly there is strong evidence at synapses demonstrating that ATP-mediated EPSCs follow from single presynaptic spikes (Edwards *et al.* 1992; Bardoni *et al.* 1997; Jo & Schlichter, 1999). We therefore anticipate that single spikes, as we have observed in a small number of cases, can also reliably release ATP from *Xenopus* neurons.

However, our results go beyond the established model of ATP cotransmission because we have discovered that the release of ATP is linked to activation of glutamate receptors, including NMDA receptors, and that this does not require spike initiation as it persists in the presence of TTX. Acetylcholine was ineffective at causing release of ATP. Although these receptors are present on spinal neurons and mediate excitation, presumably they are not localized closely enough to the sites of ATP release to be effective at inducing release. Similarly, high K^+ was never observed to cause ATP release in the presence of TTX. We suggest this treatment may be unable to cause sufficient depolarization at the sites of ATP release. Our evidence therefore suggests that glutamate receptors must be selectively localized sufficiently close to the ATP release sites so that the local depolarization and Ca^{2+} influx resulting from their activation can evoke ATP release.

Our demonstration that Cd^{2+} blocks the release of ATP evoked by glutamate suggests an involvement of HVA Ca^{2+} channels. However, the application of selective HVA channel

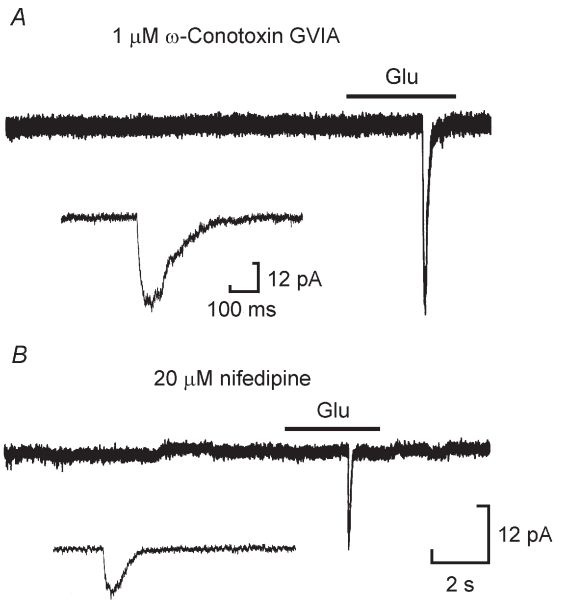

Figure 7. ATP release can occur when N-type or L-type channels are blocked

A, in the presence of ω-conotoxin GVIA, glutamate can evoke ATP release from an isolated cord. Inset shows expanded portion of the sniffer current record. *B*, nifedipine is also unable to block glutamate-induced ATP release from an isolated spinal cord (different experiment to that in *A*). Inset shows expanded portion of the response (at the same scale as the inset in *A*). Sniffer cells used in *A* and *B*.

blockers reveals interesting differences between the dependence of ATP release and synaptic transmission on the subtypes of HVA channel. Blockade of N-type channels by ω-CgTx blocks about 70 % of synaptic transmission (Wall & Dale, 1994). Although ω-CgTx may reduce the chances of observing ATP release, this effect was not statistically significant. Furthermore when ATP release was observed in the presence of ω-CgTx, it was of large amplitude. The most conservative interpretation of our results is, therefore, that release of ATP at some sites is independent of the N-type channels. Perhaps unsurprisingly, given their small contribution to the total HVA current, blockade of L-channels had no effect on glutamate-evoked ATP release. We suggest that the presently unidentifiable class of Ca^{2+} channel that comprises around 30 % of the HVA current may make an important contribution to the spike-independent release of ATP from *Xenopus* neurons.

Location of ATP release sites

The conventional view is that ATP is released from presynaptic terminals either as a principal transmitter or as a cotransmitter from the same sites and possibly from the same vesicles as the principal transmitter. Indeed there is strong evidence to support this model (Evans *et al.* 1992; Edwards *et al.* 1992; Silinsky & Redman, 1996; Bardoni *et al.* 1997; Jo & Schlichter, 1999). Nevertheless, certain aspects of our evidence suggest that ATP could also be released from locations that are distinct from the presynaptic terminal.

Firstly, the neurites of the dissociated neurons are more likely to be dendrites rather than axons (where the synaptic release sites would be located). Secondly the dependence of ATP release on the HVA channel subtypes differs from that of synaptic transmission as blockade of N-type channels cannot markedly affect ATP release. Thirdly, the release of ATP diminishes. This transient nature of ATP release cannot be simply explained by desensitization of the $P2X_2$ receptors (see Figs 1*A* and 6*A*). Desensitization of glutamate receptors might contribute to the transience of ATP release but cannot explain why it was hard to obtain repeated episodes of release as any desensitization would have been removed by the extensive rest periods given between glutamate applications. We are forced to conclude that the ATP stores themselves may be limited and largely depleted by the large release events that both spike trains and glutamate evoke. Given that the spinal circuits for motor pattern generation can function for several minutes at a time (Dale & Gilday, 1996), we think it unlikely that the stores of the primary transmitters are rapidly depleted.

While none of these observations by themselves is conclusive, together they are highly suggestive that the release of ATP can differ from that of the principal transmitter. Some sites of ATP release may be distinct from the presynaptic terminal and could possibly be located on dendrites (where glutamate receptors are predominantly located). A precedent for this latter type of mechanism has already been set with the observation that glutamate can be released from dendrites (Zilberter, 2000). An important point to note is that ATP does not act as a fast transmitter in the motor circuits of the *Xenopus* spinal cord. Instead it acts exclusively through P2Y receptors to modulate cellular properties and circuit operation. This may account for the fact that the properties of ATP release differ in this system from those that have been so far described involving the synaptic activation of P2X receptors.

Implications for signalling by ATP

While ATP can act as a transmitter in the manner of glutamate or acetylcholine, our results suggest an additional and new paradigm for ATP signalling. In addition to being released in response to action potentials, ATP release could occur as an adjunct to glutamatergic transmission in many areas of the central nervous system. Note that this is a different concept from a conventional view of cotransmission where the two transmitters are co-released. Our observations suggest that ATP is released as a downstream consequence of glutamate release and subsequent activation of receptors. Given the widespread distribution of P2X and P2Y receptors, along with ectonucleotidases, in the brain (Zimmerman, 1996; Ralevic & Burnstock, 1998) this could impact on many areas of neural function. We propose that this glutamate-dependent mechanism of ATP release could provide a way of monitoring neural activity through either the build-up of ATP, or more likely its breakdown product adenosine, in the extracellular space. This would constitute a form of volume regulation or an integrative counter.

What mechanistic advantages might accrue from linking ATP release to the activation of glutamate receptors as opposed only to action potentials? Firstly, this link may allow the release of ATP from a greater range of sites on the neuronal surface (following the distribution of the glutamate receptors). More importantly, this link changes the relationship between temporal activity of the presynaptic neuron and ATP release. Although the precise nature of this temporal relationship remains to be determined, some plausible possibilities are worth outlining. Imagine that ATP release was linked only to action potential firing. The higher the rate of firing, the more release would result, and the pattern of firing (e.g. bursting *versus* random firing with the same mean frequency) would have little effect on the amount of ATP released. Contrast this with the case when ATP release is also linked to glutamate receptor activation. Because the occupancy and activation of glutamate receptors will saturate, the pattern of synaptic activation will profoundly alter the amount of ATP released even if the mean frequency of inputs remains constant. For example, burst firing will be more effective at saturating the receptor complex and thus paradoxically will be less effective at releasing ATP than sporadic firing

with the same mean frequency. The implication of this is that highly synchronized firing may release less ATP than desynchronized activity.

ATP release and motor pattern generation in spinal cord

In vertebrate central pattern generators, glutamate acting at NMDA and AMPA receptors plays a central role in generating the motor rhythm (Grillner *et al.* 1981; Dale & Roberts, 1984, 1985; Dale & Grillner, 1986; Dale, 1986). In *Xenopus* recurrent excitatory connections between glutamatergic interneurons are particularly important for the generation and maintenance of the motor pattern (Dale & Roberts, 1984, 1985; Roberts *et al.* 1998). The very transmitter that allows initiation of the next cycle through recurrent excitation also causes, through the release of ATP and its subsequent conversion to adenosine, the slowing and final termination of the motor pattern. The purinergic modulation of spinal circuits is thus built into the central pattern generator at the most fundamental level and must be considered an intrinsic part of operation of the circuit.

Since all neurons of the motor circuit possess glutamate receptors, which are activated during swimming, any type of neuron could potentially release ATP by this mechanism. Indeed our evidence strongly suggests that the glycinergic inhibitory interneurons that play a key role in the operation of the motor circuits (Soffe & Roberts, 1982; Soffe *et al.* 1984; Dale, 1985) release ATP. Other types of neuron, which could be either excitatory interneurons or motoneurons also release ATP. Since ATP release acts as a regulatory signal that tracks the amount of activity in the motor circuits and ultimately causes their run-down and termination, there are clear mechanistic advantages to monitoring activity from all types of component neuron rather than just one subtype.

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