Calcium Flux Through Predominantly Independent Purinergic ATP and Nicotinic Acetylcholine Receptors

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Rogers, Marc, Lorna M. Colquhoun, James W. Patrick, and John A. Dani. Calcium flux through predominantly independent purinergic ATP and nicotinic acetylcholine receptors. J. Neurophysiol. 77: 1407–1417, 1997. Ligand-gated nicotinic acetylcholine receptors (nAChRs) and purinergic ATP receptors are often expressed in the same peripheral and central neurons, and ATP and acetylcholine (ACh) are stored together in some synaptic vesicles. Evidence has suggested that nAChRs and ATP receptors are not independent and that some agonists strongly cross-activate and desensitize both receptor types. Rat sympathetic neurons and nAChRs expressed in Xenopus oocytes were studied to determine the significance of the interactions caused by the two agonist types. Current amplitudes induced with separate or combined applications of ATP and nicotine are >90% additive and independent. Half of all neurons tested responded to either ATP or nicotine but not to both, indicating differences in the expression of the two receptors. In neurons that expressed both receptor types, the nAChRs were inhibited by the activity-dependent open-channel blocker chlorisondamine. If the purinergic and nicotinic receptors were significantly dependent and coactivated, then blocking the ion channels opened by a nicotinic agonist should diminish the current activated by a purinergic agonist. That result was not seen; rather, complete open-channel block of nAChRs with chlorisondamine did not significantly alter the amplitude or kinetics of ATP-induced currents in the same neurons. Finally, when cloned nAChR subunits were expressed in oocytes, ATP activated only very small currents compared with the current activated by ACh. For the 13 different nAChR subunit combinations that were studied, ATP (50–500 μM) activated a current that ranged from 0 to 4% of the size of the current activated by 100 μM ACh. In summary, we find that there is little cross reactivity, and nAChRs and purinergic ATP receptors are predominately independent, acting with separable physiological characteristics. Therefore the quantitative Ca\(^{2+}\) influx through neuronal nAChRs and ATP receptors could be separately determined for nAChRs and ATP receptors. The fraction of total current that is carried by Ca\(^{2+}\) was quantitatively determined by simultaneously measuring the whole cell current and the associated change in intracellular Ca\(^{2+}\) with fura-2. For sympathetic neurons bathed in 2.5 mM Ca\(^{2+}\) at a holding potential of −50 mV, Ca\(^{2+}\) carries 4.8 ± 0.3% (mean ± SE) of the inward current through neuronal nAChRs and 6.5 ± 0.1% of the current through purinergic ATP receptors. In conclusion, activity-dependent Ca\(^{2+}\) influx through predominately independent populations of nAChRs and ATP receptors can produce different intracellular signals at purinergic and cholinergic synapses.

INTRODUCTION

The ionotropic purinergic ATP receptors and nicotinic acetylcholine receptors (nAChRs) are both weakly selective cationic channels that are permeable to Ca\(^{2+}\) (Adams et al. 1980; Bean 1990b; Benham and Tsien 1987; Decker and Dani 1990; Fieber and Adams 1991ab; Nakagawa et al. 1990; Séguela et al. 1993; Vernino et al. 1992). The Ca\(^{2+}\) permeability of these receptors is important because activity-dependent Ca\(^{2+}\) signals mediated by these receptors are likely to serve as intracellular messengers that modulate ion channels and alter synaptic function (Fuchs and Morrow 1992; Gray et al. 1996; McGehee et al. 1995; Vernino et al. 1992). Although permeability ratios of Ca\(^{2+}\) to Na\(^{+}\) have been measured many times, those measurements do not have a strong theoretical basis for accurately predicting the Ca\(^{2+}\) flux through the channels (see Hille 1975; Vernino et al. 1994). Permeability ratios are calculated from the Goldman-Hodgkin-Katz equations, but those equations do not completely describe ion permeation in biological channels. Therefore we measured the quantitative Ca\(^{2+}\) flux through ATP- and acetylcholine (ACh)-activated channels to provide an accurate value for predicting activity-dependent intracellular Ca\(^{2+}\) signals.

To measure separately the Ca\(^{2+}\) flux through ATP receptors and nAChRs, we had to consider another issue. It is thought that ATP may potentiate nicotinic currents, and there is some controversy about whether ATP alone can activate nAChRs (Bean 1992; Igusa 1988; Lu and Smith 1991). Recently it was reported that the current obtained by adding the contributions activated separately by ACh and ATP was larger than current activated by the simultaneous application of ATP and ACh; the currents were not additive (Nakazawa et al. 1994; Nakazawa et al. 1991). Those results led to the hypothesis that ATP opens a subpopulation of ACh-activated channels, but the conducting state opened by ATP is different from that opened by ACh. That hypothesis has important implications because ATP is sometimes coreleased with ACh, and many postsynaptic cells express both ATP and nAChR channels, including skeletal muscle (Igusa 1988), hair cells (Housley et al. 1992), PC12 cells, peripheral ganglia neurons (Evans et al. 1992; Fieber and Adams 1991ab; Nakazawa et al. 1991; Silinsky and Gerzanich 1993), and central neurons (Edwards et al. 1992; Nabekura et al. 1995).

There is evidence for interaction between the two transmitter systems: in some cases there is potentiation and in other cases inhibition (Akasu and Koketsu 1985; Fu 1994; Igusa 1988; Lu and Smith 1991; Mozrzymas and Ruzzier 1992; Nakazawa 1994). In our hands, however, the ATP-activated currents in sympathetic neurons isolated from rat superior cervical ganglia (SCG) do not behave as a subset of the ACh-activated currents. This result is compatible with the recent cloning of purinergic receptors from smooth muscle (Valera et al. 1994), peripheral neurons (Brake et al. 1994; Chen et al. 1995; Lewis et al. 1995), and brain (Buell et al. 1996; Séguela et al. 1995), showing that purinergic
receptors belong to a new class of ionotropic receptor with similarities to inward rectifier K⁺ channels and epithelial Na⁺ channels (Surprenant et al. 1995). The cloned nAChR subunits, on the other hand, share similarities with ligand-gated γ-aminobutyric acid and glycine receptor channels (Betz 1991). We show here that there is a small amount of interaction and that ATP receptors and nAChRs are predominantly independent and contribute separable Ca²⁺ fluxes in rat sympathetic SCG neurons.

**METHODS**

**Cell culture**

Neuronal purinergic ATP and nAChR channels were studied in sympathetic neurons isolated from rat SCG with the use of methods similar to those of Mathie et al. (1987). SCG were removed from anesthetized Sprague-Dawley rats of postnatal day 18–27. The connective tissue sheath was removed and ganglia fragments were incubated for 30–40 min with 250 U/ml collagenase (Worthington Biochemical, Freehold, NJ) and 150 U/ml DNase (Sigma Chemical, St Louis, MO) in Dulbecco’s modified Eagle’s Medium (DMEM; Gibco BRL, Grand Island, NY). The supernatant was removed and the ganglia were incubated for 30 min in a solution of DMEM with 100 U/ml trypsin (Sigma) and a 50% dilution of the collagenase and DNase solution. Ganglia were washed in DMEM containing 10% fetal bovine serum (FBS) to inactivate the trypsin and then triturated through a series of flamed Pasteur pipettes before being resuspended in DMEM containing 10% FBS and 10–25 ng/ml nerve growth factor (Collaborative Research, Bedford, MA).

Sympathetic neurons were plated into culture dishes containing coverslips coated with collagen (Sigma) and poly-d-lysine (Collaborative Research) and were osmotically adjusted to 300 mosM with sucrose. All external solutions contained 0.5 μM atropine and 250 mM TTX to inhibit muscarinic receptors and voltage-dependent Na⁺ channels. Nicotine (100 μM, Sigma) and ATP (100–500 μM of Na⁺ salt, Sigma) were made up daily from frozen stock solutions. Zn²⁺ (10 μM) was added to ATP agonist solutions to maximally potentiate the current amplitudes (Cloues et al. 1993).

These experimental conditions ensured that agonist currents were only due to ion flux through the ligand-gated channel of interest, and the internal solution of impermanent ions ensured that receptor currents were all unidirectional, inward currents (see Vernino et al. 1994). Recordings were chosen to isolate ligand-gated receptor currents from other membrane conductances. Pipette solutions contained (in mM) 140 Na-CH₃SO₃, 1 Mg-ATP, 5 tetraethylammonium chloride (TEA), 1 fura-2, and 20 HEPES. The solution was adjusted to pH 7.4 with methanesulfonic acid, which provides the major anion. Extracellular test solutions contained (in mM) 150 NaCl, 2.5 CaCl₂, and 10 HEPES. The extracellular ‘pure’ Ca²⁺ solution contained 75 mM CaCl₂ and 10 mM HEPES. Solutions were adjusted to pH 7.4 with the hydroxide of the major cation and were osmotically adjusted to 300 mosM with sucrose. All external solutions contained 0.5 μM atropine and 250 mM TTX to inhibit muscarinic receptors and voltage-dependent Na⁺ channels. Nicotine (100 μM, Sigma) and ATP (100–500 μM of Na⁺ salt, Sigma) were made up daily from frozen stock solutions. Zn²⁺ (10 μM) was added to ATP agonist solutions to maximally potentiate the current amplitudes (Cloues et al. 1993).

For simple current measurements without microfluorimetry, the experiment with the use of a microforge (Narishige USA). For compound currents measurements without microfluorimetry, the internal solution in the patch pipettes contained (in mM) 130 Cs-CH₂SO₃, 5 NaATP, 5–20 NaCl, 10 ethylene glycol-bis(β-aminoethyl ether)-N,N',N″,N‴-tetraacetic acid (EGTA) and/or bis-(o-aminophenoxy)-N,N″,N‴,N‴′-tetraacetic acid (BAPTA), and 10 N-2-hydroxyethylpipеразине-N'-2-ethanesulfonic acid (HEPES). Extracellular solutions contained (in mM) 150 Na-CH₂SO₃, 1 CaCl₂, and 10 HEPES. Solutions were adjusted to pH 7.4 with methanesulfonic acid, and the osmolality was adjusted with sucrose to 295 mosM (pipette) or 315 (external) mosM. All external solutions also contained 0.5 μM atropine (Sigma) to inhibit muscarinic receptors and 250 mM tetrodotoxin (TTX) (Calbiochem) to block voltage-dependent Na⁺ channels. Voltage-dependent and Ca²⁺-activated K⁺ channels were inhibited by replacing intracellular K⁺ with Cs⁺, whereas Ca²⁺-activated Cl⁻ conductances were eliminated by replacing Cl⁻ with the impermeant anion methanesulfonate. Fast agonist and drug applications were made with large outflow tubes positioned in a row and mounted on a computer-controlled high-speed drive (Newport, Irvine, CA) (Vernino et al. 1992, 1994).

Drug solutions were made up fresh daily from frozen stock solutions. Final concentrations were as follows: nicotine, 100 μM; Na-ATP, 100–500 μM; adenosine, 10 μM; AMP, 500 μM; α,β-methylene ATP (α,β-MeATP), 100–250 μM. Reactive Blue 2 (10 μM) and chlorisondamine (chlsdm) (5–100 μM) were all obtained from Sigma. Suramin (25–400 μM) was from CB Chemicals (Woodbury, CT).

**Fura-2 microfluorimetry**

To determine the quantitative influx of Ca²⁺ through the ATP receptors and nAChRs, we simultaneously measured current and the change in intracellular Ca²⁺ with fura-2 following the techniques described in detail by Vernino et al. (1994). Recording solutions were chosen to isolate ligand-gated receptor currents from other membrane conductances. Pipette solutions contained (in mM) 140 N-methyl-d-glucamine, 1 Mg-ATP, 5 tetraethylammonium chloride (TEA), 1 fura-2, and 20 HEPES. The solution was adjusted to pH 7.4 with methanesulfonic acid, which provides the major anion. Extracellular test solutions contained (in mM) 150 NaCl, 2.5 CaCl₂, and 10 HEPES. The extracellular ‘pure’ Ca²⁺ solution contained 75 mM CaCl₂ and 10 mM HEPES. Solutions were adjusted to pH 7.4 with the hydroxide of the major cation and were osmotically adjusted to 300 mosM with sucrose. All external solutions contained 0.5 μM atropine and 250 mM TTX to inhibit muscarinic receptors and voltage-dependent Na⁺ channels. Nicotine (100 μM, Sigma) and ATP (100–500 μM of Na⁺ salt, Sigma) were made up daily from frozen stock solutions. Zn²⁺ (10 μM) was added to ATP agonist solutions to maximally potentiate the current amplitudes (Cloues et al. 1993).

These experimental conditions ensured that agonist currents were only due to ion flux through the ligand-gated channel of interest, and the internal solution of impermanent ions ensured that receptor currents were all unidirectional, inward currents (see Vernino et al. 1994). Voltage-dependent and Ca²⁺-dependent channels were inhibited by voltage clamping at a constant negative holding potential and by the ionic composition of the internal and external solutions. K⁺ channels were inhibited by replacing intracellular K⁺ and including TEA. To ensure that Ca²⁺ signals were not from intracellular stores, 0.1–0.3 μM thapsigargin was added fresh daily to the pipette solution to inhibit Ca²⁺ uptake into intracellular stores. In addition, cells were perfused with 10 mM caffeine in zero Ca²⁺ to release Ca²⁺ from internal stores while refilling was inhibited, thereby removing any Ca²⁺-induced Ca²⁺ release component from the fura-2 signals.

Simultaneous measurements of the intracellular calcium concentration and membrane current were made at the single-cell level with the use of fura-2 microfluorimetry and whole cell patch-clamp techniques, as we described previously (Vernino et al. 1994). The cell-impermeant pentapotassium form of fura-2 (Molecular Probes, Eugene, OR) was allowed to equilibrate within a cell before measurements of intracellular Ca²⁺ were made. The excitation wave-length rapidly alternated between 340 and 380 nm, and the fura-2 fluorescence emission was collected at >480 nm with a photomultiplier (Thor EMI, Middlesex, UK). Free Ca²⁺ concentration was calculated with the use of the equation of Grynkiewicz et al. (1985): 

\[ [Ca^{2+}] = K(R - R_{min})/(R_{max} - R) \]

where R is the ratio of fura-2 fluorescence measured every 17 ms. Calibrations of the microfluorimetry setup with Ca²⁺ standards gave values for K, R_{min}, and R_{max}.
and $R_{\text{max}}$ of 2.0 $\mu M$, 0.3, and 4. A high intracellular concentration of 1 mM fura-2 was used so that the indicator would overwhelm endogenous cytosolic buffers and more accurately indicate all the Ca$^{2+}$ influx across the membrane (see Neher and Augustine 1992; Vernino et al. 1994; Zhou and Neher 1993).

**Expression of nAChR subunits in Xenopus oocytes**

Oocytes were surgically removed from adult *Xenopus laevis* frogs and were treated with 0.75 mg/ml collagenase A (Boehringer) for 2 h at room temperature. The oocytes were manually defolliculated and injected with combinations of rat nAChR subunit cDNAs encoded in PSM plasmid vector. Each oocyte was injected with an average of 10–15 ng DNA. Oocytes were incubated at 19°C for 2–7 days in Barth’s solution containing 10 $\mu g$/ml gentamicin. For current recordings, oocytes were placed in a 300-$\mu l$ chamber at room temperature and perfused at a rate of 10 ml/min with the following bath solution: 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl$_2$, 1.8 mM MgCl$_2$, and 10 mM HEPES, pH 7.2, and 1 $\mu M$ atropine. The oocytes were voltage clamped and held at −60 mV with the use of an Axoclamp 2A amplifier. Drugs were applied via a large inflow port controlled by solenoid switching valves. Currents were recorded to an Apple MacIntosh IIci hard disk with the use of the Labview II data acquisition virtual instrument.

**RESULTS**

**ATP-activated currents have P2 purinoceptor characteristics**

ATP elicited rapidly activating currents from rat SCG neurons. Because P2 purinoceptors on SCG neurons are less responsive than in other neurons (Khakh et al. 1995), we used a saturating concentration of ATP (500 $\mu M$) and 10 $\mu M Zn^{2+}$ to potentiate the ATP receptor currents maximally (Cloues et al. 1993). The pharmacology of purinergic ATP receptors remains unclear, but the cloning of ionotropic and metabotropic receptors has revealed some ligands for differentiating between receptor subtypes (Fredholm et al. 1994). Rapidly activating inward currents were only produced in rat sympathetic neurons by ATP and the nonhydrolyzable analogue $\alpha,\beta$-MeATP (Fig. 1A). Consistent with previous results (Cloues et al. 1993), we found that only a fraction of neurons (3 of 7) that responded robustly to ATP also responded to $\alpha,\beta$-MeATP (100–250 $\mu M$), and this ligand always produced smaller currents. ATP is rapidly hydrolyzed by ecto-ATPases and some breakdown products also activate P1 receptors; however, no membrane currents were seen on those obtained by Bean (1990a) in bullfrog dorsal root ganglion neurons.

The agonist pharmacology was verified with suramin, which is a competitive antagonist at P2 purinoceptors (Hoyle et al. 1990). Rapid coapplication of suramin and ATP reduced the current amplitude compared with ATP applied alone to the same neuron (Fig. 1B). Inhibition of ATP receptor currents by suramin was dose dependent and was only slowly reversible. It has been shown previously by Nakazawa (1994) that suramin does not inhibit nAChR receptors.

Other characteristic of the ATP-induced currents also indicated that they arose from ligand-activated ATP purinoceptors. Inward current rapidly reached a maximum, and the currents decayed back to the baseline in ~2 s after the agonist was removed (Fig. 2A). Both the time course and the voltage dependence of the currents are inconsistent with metabotropic receptors. Also, metabotropic receptor activation involves G proteins, but the ATP-elicited currents were unaffected by inclusion of guanosine 5’-O-(3-thiotriphosphate) (GTP-γ-S) or guanosine 5’-O-(2-thiodiphosphate) (GDP-β-S) in the patch pipettes ($n = 5$). Furthermore, metabotropic ATP receptors mobilize Ca$^{2+}$ from internal stores, leading to the activation of a nonspecific cation conductance. The ATP-activated currents we studied were not directly dependent on intracellular Ca$^{2+}$, because they could be elicited when the neurons were pretreated with caffeine and thapsigargin to discharge internal Ca$^{2+}$ stores (Fig. 7) and when the patch pipette contained 10 mM EGTA and/or BAPTA to chelate intracellular Ca$^{2+}$. Finally, metabotropic ATP receptors were ruled out because ATP-induced currents measured in Ca$^{2+}$-free solutions did not show fura-2 Ca$^{2+}$ transients, which would have been seen if metabotropic ATP receptors had caused the release of Ca$^{2+}$ from intracellular stores.

**Differences in the expression and characteristics of purinergic and nicotinic currents**

It has been suggested that ATP may interact with nAChRs, either as a modulator or by directly opening nicotinic receptors (reviewed by Bean 1992). It also has been hypothesized that ATP may function only to activate a subset of nAChRs on rat sympathetic neurons and PC12 cells (Nakazawa 1994; Nakazawa et al. 1991). Before measuring the Ca$^{2+}$ flux through the ATP receptors and nAChRs, we examined the extent of interaction between the two neurotransmitters.

The first indication that the receptor types are independent is that we observed differences in expression of ATP and nAChR currents in rat sympathetic neurons. When 11 neurons were sequentially exposed to ATP and nicotine, ATP elicited currents in 8 and nicotine induced currents in 7, but only 4 of 11 neurons showed a response to both agonists. Three of the neurons responding to nicotine did not respond to ATP (500 $\mu M$) and Zn$^{2+}$ (10 $\mu M$), and four neurons responding to ATP failed to exhibit nicotine-induced (100 $\mu M$) currents. Additionally, there was not a direct correlation between peak ATP receptor and nAChR currents in neurons that responded to both agonists. These results are similar to those obtained by Bean (1990a) in bullfrog dorsal root ganglion neurons.

There were marked differences in the characteristics of macroscopic currents evoked by nicotine and ATP in rat sympathetic neurons. Rapid application of moderate concentrations of nicotine generally produced desensitizing currents, but even relatively high concentrations of ATP produced little rapid desensitization, even during applications for $>10$ s (Fig. 2 and data not shown). In Fig. 2, the average current-voltage relationships for nAChR ($n = 5$) and purinergic ATP receptor channels ($n = 7$) are compared. The apparent reversal potential for the nicotinic currents is −2 mV and that for the purinergic currents is 3 mV. All currents were recorded with the same cesium-based internal solution and sodium-based external solution containing 1 mM Ca$^{2+}$.

**Additivity of nAChR and ATP receptor currents**

Previously it was found that the peak nicotinic and purinergic currents activated separately summed to a larger
ATP receptor currents have P2 purinergic pharmacology. A: agonist pharmacology. Application of Na-ATP (500 μM) and α,β-methylene ATP (α,β-MeATP) (100 μM) elicit currents in a rat sympathetic neuron; adenosine monophosphate (AMP, 500 μM) does not. In another cell, rapid application of adenosine (10 μM) failed to activate current. B: suramin (100 μM) coapplied with ATP (100 μM) partially inhibits the current. Inhibition of ATP-evoked current by suramin was dose dependent (right), with substantial reduction at 25 μM, increasing to ~80% at 400 μM.

Either ATP (500 μM) or nicotine (100 μM) was rapidly applied to neurons. After several applications of one agonist, a stable amplitude was recorded; then the other agonist was applied until the current amplitude reached a steady level (Fig. 3A). Finally, a mixture of ATP and nicotine was applied. This pattern of agonist applications was repeated and reversed several times during the course of an experiment.

We invariably found that nAChR and ATP receptor currents were almost completely additive. Figure 3B shows results from a typical cell; it can be seen that the numerical sum of the currents elicited with ATP or nicotine alone (∙∙∙) closely predicts the amplitude of the membrane current recorded in response to coapplication of ATP and nicotine (——). Currents elicited by ATP showed fast activation but little desensitization, whereas currents evoked by nicotine exhibited a rapid peak followed by a decay. The numerical sum of these currents shows a fast peak with some desensitization, but the actual current elicited by coapplication of ATP and nicotine appears to have a slower activating peak and slightly less decay. Therefore measurements of peak current amplitude could underestimate the degree of additivity, but calculation of the total current flowing (charge integral) may better reflect the true situation. In light of this, we calculated the additivity between ATP and nAChR currents with the use of both methods (Fig. 3C). In four cells, there was 89.7 ± 0.9% (mean ± SE) additivity of peak current amplitude, and there was 99.9 ± 3.5% additivity for the integral of the current (charge transferred) during the simultaneous opening of purinergic and nicotinic receptors. The small differences in the kinetics and additivity suggest there could be some modulatory effects, but the first-order conclusion is that the currents are predominantly independent and additive.

ATP receptors and nAChRs are predominantly independent populations on rat SCG neurons

We conducted experiments with the open-channel blocker chlsdm to investigate more rigorously the hypothesis that ATP activates a subpopulation of nAChRs, as well as ligand-gated purinergic receptors. Chlsdm enters nAChR channels only after they open, and then chlsdm becomes trapped within the pores as they close (Amador and Dani 1995; Neely and Lingle 1986). The block is relieved only after the nAChRs are reopened and depolarizing voltages are used to drive chlsdm out of the pores. Closed nAChR channels
are not affected by chlsdm (Amador and Dani 1995; Neely and Lingle 1986).

Figure 4 shows that ATP-induced currents are only very slightly changed by completely blocking all the nAChRs with chlsdm. First, ATP (500 μM) was applied to induce a purinergic current (Fig. 4A). Then nicotine (100 μM) was applied repeatedly in the presence of 5 μM chlsdm until all the nAChRs opened and were progressively blocked (Fig. 4B). Between 6 and 12 applications of nicotine plus chlsdm produced complete block of nAChRs. After the chlsdm was briefly washed away, ATP was again applied, producing a current nearly identical to the current before blockade of the nAChRs (Fig. 4C). There was no significant change in the amplitude or time course of whole cell ATP currents after complete block of nAChRs. In five neurons, the ATP current measured after chlsdm treatment was only 3.0 ± 1.5% smaller in peak amplitude than during the first application of ATP (P > 0.5).

A related issue to consider is that ATP could relieve the block by chlsdm if ATP opened the same channels that had been blocked during the nicotine applications. In that case, if ATP opened the blocked channels, chlsdm would significantly unblock; and nicotine could then activate a current. Figure 4D shows that, after an ATP application, nicotine (with chlsdm) activates a very small current that is again blocked by chlsdm after several applications of nicotine. This much relief of the block is expected even without opening the receptors (see Amador and Dani 1995). A third application of ATP (Fig. 4E) again produces a similar-sized current. For the three neurons in which the experiment in Fig. 4 could be completed, the third ATP current was reduced in amplitude by an average of 10.4 ± 1.0% compared with the first current. At a maximum, only a few percent of the ATP-activated current is contributed by nAChRs.

**ATP does not significantly activate heterologously expressed nAChRs**

Our results with SCG neurons indicate that nicotinic ACh and ATP act almost completely on independent receptors. It is possible, however, that another ganglionic subtype of nAChR (not significantly expressed in the neurons we studied) could be more sensitive to ATP. To test this possibility, various α- and β-subunits of the nAChR family were expressed in Xenopus oocytes. The mRNA coding for many of these subunits is expressed in rat sympathetic neurons (Mandelzys et al. 1994).

Some combinations of cDNA injected into Xenopus oocytes, including α3, β2, and β3, produced functional receptors that responded to moderate concentrations of ACh (30–100 μM) with large currents (Table 1). ATP (50–500 μM) and α,β-MeATP (50–500 μM) also were applied to the oocytes, but no significant current responses were seen. The largest currents activated by ATP were measured in oocytes injected with α3β2β3. Only 4 of 11 of the oocytes coinjected with β3 produced significant current in response to ATP, and that current corresponded on average to 2.0 ± 0.5% of the current induced by ACh. The more typical response, however, is shown in Fig. 5, where 100 μM ACh produced a large current; but 500 μM ATP or α,β-MeATP produced no detectable current at the same gain. Addition of α5 to subunit combinations that formed functional nAChRs did not alter the responses to ACh or ATP. The α5 subunit can contribute to nAChRs (Conroy and Berg 1995; Conroy et al. 1992; Ramirez-Latorre et al. 1996; Wang et al. 1996), but our rat α5, α6, and β3 subunits did not produce significant numbers of functional nAChRs when expressed alone in oocytes or as the sole α- or β-subunit in pairs with other subunits.

**Quantitative fraction of the current carried by Ca\(^{2+}\) through nAChRs and ATP receptors**

The fraction of the current carried by Ca\(^{2+}\) was determined with the use of methods we developed previously (Rogers and Dani 1995; Vernino et al. 1994). Rat SCG neurons were whole cell voltage clamped, and the change in the concentration of intracellular Ca\(^{2+}\) (Δ[Ca\(^{2+}\)]) was monitored with fura-2 microfluorimetry. The fraction of current carried by Ca\(^{2+}\) was determined by comparing simultaneous records of current and Δ[Ca\(^{2+}\)] in a physiological solution of Na\(^+\) and Ca\(^{2+}\) with those obtained in pure external Ca\(^{2+}\).
FIG. 3. ATP and nicotinic currents are almost completely additive. A: rapid application of ATP (500 μM) elicits a current, whereas application of nicotine (100 μM) to the same neuron produces a larger, desensitizing current. Intervals of 30–60 s between agonist applications avoided longer-term desensitization. Repeated applications led to stable current amplitudes at a holding potential of ~50 mV. Subsequent coapplication of the same concentrations of ATP and nicotine evokes a larger, moderately desensitizing current. B: coapplication of ATP and nicotine (I\text{ATP} + I_{\text{Nic}}) (——) compared with the computed sum of the currents (I_{\text{ATP}} + I_{\text{Nic}}) (······). C: averaged data (n = 4) comparing the additivity: I_{\text{ATP}} + I_{\text{Nic}} compared with I_{\text{ATP}} + I_{\text{Nic}} peak currents or the integrated current (charge).

FIG. 4. Open-channel blockade of nicotinic acetylcholine receptors (nAChRs) indicates ATP activates a predominantly separate population of receptors. A: ATP (500 μM) was applied to a rat sympathetic neuron. B: nicotine (100 μM) + chlorisondamine (Chlsdm) (5 μM) were repeatedly applied to allow chlsdm to enter and block the nAChRs after they opened. C: ATP-activated current was practically unchanged. D: nicotine + chlsdm currents were mostly still blocked. The small recovery was blocked after several applications. E: another application of ATP activated a similar current. Scale bars: 50 pA (ATP currents), 100 pA (nicotinic currents). Holding potential: ~50 mV.

In pure external Ca\textsuperscript{2+}, the inward current through the receptor channel is carried exclusively by Ca\textsuperscript{2+}. Therefore a particular fura-2 response can be associated with a known Ca\textsuperscript{2+} influx. This association serves as a phenomenological calibration of the fura-2 response to Ca\textsuperscript{2+} for each cell, and the magnitude of Δ[Ca\textsuperscript{2+}], in a physiological solution indicates how much of that current was carried by Ca\textsuperscript{2+}.

Simultaneous recordings of the current and the Δ[Ca\textsuperscript{2+}], elicited by nicotine are shown in physiological solution containing 2.5 mM Ca\textsuperscript{2+} (Fig. 6A) and in pure external Ca\textsuperscript{2+} (Fig. 6B). In Fig. 6C the Δ[Ca\textsuperscript{2+}], is plotted against the integral of the membrane current (charge). By dividing the slope of the Δ[Ca\textsuperscript{2+}], versus charge plot in pure Ca\textsuperscript{2+} into that in physiological solution (Fig. 6C, ------), the percent-
**TABLE 1. Neuronal nAChR subunits expressed in Xenopus oocytes**

<table>
<thead>
<tr>
<th>Subunit Combination</th>
<th>ACh</th>
<th>ATP</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>α3β2/δ4</td>
<td>1.570 ± 325</td>
<td>5 ± 1</td>
<td>23</td>
</tr>
<tr>
<td>α3β2</td>
<td>919 ± 156</td>
<td>7 ± 2</td>
<td>3</td>
</tr>
<tr>
<td>α3δ4</td>
<td>616 ± 143</td>
<td>8 ± 4</td>
<td>5</td>
</tr>
<tr>
<td>α3β2/δ3</td>
<td>676 ± 289</td>
<td>13 ± 11</td>
<td>8</td>
</tr>
<tr>
<td>α3β4/δ3</td>
<td>1.423 ± 95</td>
<td>22 ± 4</td>
<td>3</td>
</tr>
<tr>
<td>α3α5/β2</td>
<td>363 ± 73</td>
<td>8 ± 3</td>
<td>3</td>
</tr>
<tr>
<td>α3α5/δ4</td>
<td>270 ± 29</td>
<td>12 ± 4</td>
<td>3</td>
</tr>
</tbody>
</table>

Inward currents at a holding potential of −60 mV are means ± SE, expressed in nA. All subunit combinations were injected in equal ratios. Expression of functional receptors was determined for every batch of oo-

cytes by application of 100 μM acetylcholine (ACh) and 50–100 μM ATP and/or α,β-methylene ATP: nAChR, nicotinic ACh receptor.

The age of current carried by Ca\(^{2+}\) was determined (see Vernino et al. 1994). In an external solution containing 2.5 mM Ca\(^{2+}\) at a holding potential of −50 mV, the average percentage of inward current carried by Ca\(^{2+}\) through neuronal nAChRs in rat sympathetic neurons is 4.8 ± 0.3% (n = 5).

Because rat sympathetic neurons have α-bungarotoxin (α-BGT) binding sites and express the gene for α7 (Mandelzys et al. 1994; Smith and Kessler 1988), it was possible that α7 receptor subunits were contributing to the Ca\(^{2+}\) influx. nAChRs containing the α7 subunit have a higher Ca\(^{2+}\) permeability (Castro and Albuquerque 1995; Séguela et al. 1993), but they can be inhibited by α-BGT. To eliminate the possibility of a contribution by α7 (or any α-BGT-sensitive receptor), the neurons were incubated in 50 nM α-BGT for >60 min to saturate available binding sites. After α-BGT treatment, the fraction of inward nAChR current carried by Ca\(^{2+}\) was the same as without the treatment: 4.8 ± 0.6% (n = 2). This result indicates that the Ca\(^{2+}\) influx we are measuring is due to α-BGT-insensitive nAChRs. If α-BGT-sensitive receptors are present, they do not contribute significantly to the current or Ca\(^{2+}\) influx caused by activating all the nAChRs by rapid, whole cell application of nicotine. This conclusion does not hold for all other types of neurons (see Gray et al. 1996).

Under identical conditions, the fraction of inward current carried by Ca\(^{2+}\) through purinergic ATP receptors was 6.5 ± 0.1% (n = 8) (Fig. 7). This value is significantly greater (P < 0.001) than the fractional Ca\(^{2+}\) influx through α-BGT-insensitive nAChRs recorded in the same SCG neurons.

It was possible that metabotropic ACh and ATP receptors may have been activated by the ligands used in these experiments. This could have elicited second-messenger-mediated Ca\(^{2+}\) release from intracellular stores without corresponding changes in membrane current, in which case the fractional Ca\(^{2+}\) flux would be inaccurately high. This possibility was tested and minimized by incubating neurons in between agonist applications in a Ca\(^{2+}\)-free external solution containing 10 mM caffeine, which releases Ca\(^{2+}\) from Ca\(^{2+}\)-sensitive intracellular stores (Lipscombe et al. 1988), whereas the Ca\(^{2+}\)-free solution prevents these stores from refilling (Thayer et al. 1988). Pipettes also contained 100–300 nM thapsigargin to inhibit the ATP-dependent pumps that refill the stores (Thastrup et al. 1990). As a further test, no increase of intracellular Ca\(^{2+}\) concentration was seen in Ca\(^{2+}\)-free external solution even after up to 15 s in agonist, arguing against activation of metabotropic receptors. Figures 6C and 7C also show a linear relationship between the Δ[Ca\(^{2+}\)] and the charge for both nAChRs and ATP receptors. A linear Δ[Ca\(^{2+}\)]-charge relationship indicates that only the influx of current is contributing to the Ca\(^{2+}\) signal (see Vernino et al. 1994).

Our previous work also enabled us to establish rigorous protocols to avoid artifacts (Rogers and Dani 1995; Vernino et al. 1994). We required that the starting intracellular Ca\(^{2+}\) concentration be low (<100 nM) and that intracellular Ca\(^{2+}\) concentration remains <400 nM for the duration of agonist currents. We also tested the validity of the linear Δ[Ca\(^{2+}\)]-charge relationship by comparing the membrane current integral with the time course of the Ca\(^{2+}\) transient. The inverse of the agonist current integral was aligned (with a slight offset) with the fura-2 records in physiological and pure external Ca\(^{2+}\) solution (see Figs. 6 and 7). In all cases, charge influx through nAChR and ATP receptors predicted the shape of the Ca\(^{2+}\) transient. This linear correlation indicates that the measured Ca\(^{2+}\) transient is a direct result of agonist-induced Ca\(^{2+}\) influx and is unlikely to be affected by Ca\(^{2+}\)-induced Ca\(^{2+}\) release from intracellular stores, transport processes, nonlinear Ca\(^{2+}\) buffering, or Ca\(^{2+}\)-activated conductances (Schmeggenburger et al. 1993; Thayer and Miller 1990; Vernino et al. 1994).

**DISCUSSION**

**ACh and ATP acted predominantly as independent agonists for separate receptors.**

The agonist and antagonist pharmacology, the kinetics of the currents, and Ca\(^{2+}\) flux characteristics of the currents all
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**FIG. 6.** Calcium influx through neuronal nAChRs. Simultaneous recordings of current (bottom) and fura-2 signal (top) are shown for a neuron (A) in a physiological solution containing 2.5 mM Ca$^{2+}$ and (B) in a pure external Ca$^{2+}$ solution. The agonist was nicotine (100 µM in A and 25 µM in B). The cell was held at −50 mV and was perfused with the impermeant internal solution. The integrated currents are shown as smooth curves, slightly displaced from the fura-2 signal to show that the shape of the integrated current follows the fura-2 signal throughout the period of agonist application. C: fraction of current carried by Ca$^{2+}$ was determined by plotting change in intracellular Ca$^{2+}$ concentration (Δ[Ca$^{2+}]_i$) vs. cationic influx (pC) in 2.5 mM Ca$^{2+}$ (○) and in pure Ca$^{2+}$ (●). In this neuron 4.7% of the current was carried by Ca$^{2+}$.

Indicate that ionotropic P2 purinergic receptors mediated the ATP-activated currents in the SCG neurons. The differences in expression, voltage dependence, cation selectivity, and Ca$^{2+}$ permeability all indicate that nAChRs and ATP receptors form predominantly independent populations on rat SCG neurons. Also, in our hands, the currents induced by nicotine and ATP alone are almost equal to the current induced by coapplication. Finally, the open-channel blocker chlsdm provides strong evidence that at most 10% of the ATP-induced current may be influenced by interaction with the nicotinic receptors. The heterologous expression of nAChRs in oocytes also indicates that there is only a slight direct activation of neuronal nAChRs by ATP.

Contrary to our results, Nakazawa (1994) found that nicotinic and purinergic currents were not additive. That conclusion arises (in part) from measurements of peak current rather than the integral of the current. The peak current requires rapid solution changes, and even then the peak current is more influenced by the slight changes in kinetics described in Fig. 3. Studies in cardiac ganglion and hair cells agree with our results, indicating no interactions between ATP and neuronal nAChRs (Fieber and Adams 1991b; Housley et al. 1992). It seems to us that the evidence is stronger that ATP may significantly activate muscle (or Torpedo) nAChRs (Fu 1994; Igusa 1988; Lu and Smith 1991; Mozrzymas and Ruzzier 1992), which appear to possess an ATP binding site of unknown function (Schrattenholz et al. 1994).

**Quantitative Ca$^{2+}$ flux through nAChRs and ATP receptors**

The fraction of the current carried by Ca$^{2+}$ through nAChRs and ATP receptors has been estimated several times with the use of related methods. In our method, a pure Ca$^{2+}$ current through the same channels in the same neuron serves as the calibration of the fura-2 signal. In a related method, voltage-dependent Ca$^{2+}$ channels (VDCCs) from different
neurons are used to obtain the calibration. Calibration with VDCCs gives a slightly smaller estimate for the fraction of current carried by Ca\(^{2+}\) through ligand-gated channels (see Rogers and Dani 1995). The advantages of using the same channels and neurons for both the test and calibration have been examined in detail previously (Rogers and Dani 1995).

When VDCCs were used for the calibration, it was estimated that ganglionic-type nAChRs carry 2.5% of the current in a physiological solution of 2 mM Ca\(^{2+}\) (Zhou and Neher 1993). With the use of the pure Ca\(^{2+}\) calibration, we estimated that 4.8% of the current is carried by Ca\(^{2+}\) through ganglionic nAChRs in 2.5 mM Ca\(^{2+}\). For ATP receptors in smooth muscle, the VDCC calibration was used to estimate that 5.1% of the current is carried by Ca\(^{2+}\) in a solution of 3.6 mM Ca\(^{2+}\) (Schneider et al. 1991). With the use of sympathetic neurons, our estimate is 6.5% for ATP receptors in 2.5 mM Ca\(^{2+}\). The studies qualitatively agree that ATP receptors carry a higher percentage of Ca\(^{2+}\) than ganglionic nAChRs.

Calcium signals at purinergic and nicotinic synapses

The role of receptor-mediated Ca\(^{2+}\) influx at nicotinic and purinergic synapses could be quite varied, because nAChRs and ATP receptors are located both pre- and postsynaptically (reviewed in McGehee and Role 1995; Sargent 1993; Zimmermann 1994). ACh mediates fast postsynaptic signals at the neuromuscular junction and in the peripheral ganglia, but fast synaptic transmission has not been found in the CNS, except at the Renshaw synapse in the spinal cord (see Katz 1966; McGehee and Role 1995; Sargent 1993). ATP mediates fast synaptic transmission at the vascular neuroeffector junction and at synapses in the peripheral nervous system and CNS (Edwards et al. 1992; Evans and Surprenant 1992; Evans et al. 1992; Galligan and Bertrand 1994). It has been shown that nicotinic and purinergic activity can produce Ca\(^{2+}\) signals that open Ca\(^{2+}\)-dependent K\(^+\) and Cl\(^-\) channels (Benham et al. 1987; Mulle et al. 1992; Tokimasa and North 1984; Vernino et al. 1992), which con-
sequentially affect neuronal activity (Fuchs and Morrow 1992; Wong and Gallagher 1991). Nicotinic and purinergic autoreceptors on nerve terminals can modulate the spontaneous and evoked release of neurotransmitters and may act to prevent synaptic fatigue (McMahon et al. 1994; Pelleg and Hurt 1996; Vizi and Somogyi 1989; Wonnacott et al. 1990). A presynaptic Ca\(^{2+}\) influx mediated by nAChRs enhances the release of glutamate in the medial habenula and the hippocampus (Gray et al. 1996; McGeehe et al. 1995). These results support the hypothesis that ATP- and ACh-mediated Ca\(^{2+}\) signals may participate in synaptic plasticity, possibly via second-messenger systems such as protein phosphorylation or arachidonic acid release (Raymond et al. 1993; Vijayaraghavan et al. 1995).

It seems likely that a complex interplay exists between synaptic activity, the intracellular Ca\(^{2+}\) signals generated by nicotinic and purinergic receptors, and the consequent modulation of synaptic properties. Although ACh and ATP can be coreleased and their receptors share synaptic locations, there may be only a slight amount of direct interaction. Rather, the two heterogeneous receptor types seem to provide separate pathways for activity-dependent Ca\(^{2+}\) signals with different kinetics, rates of desensitization, and abilities to mediate Ca\(^{2+}\) influx. These features may be important for the roles of nicotinic and purinergic ligand-gated receptors in synaptic development, maintenance, and plasticity.

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