Comparison of Quantitative Calcium Flux through NMDA, ATP, and ACh Receptor Channels

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ABSTRACT NMDA receptors, ATP receptors, and nicotinic ACh receptors respond to agonist by undergoing conformational changes that open weakly selective cationic channels that are permeable to calcium. We determined the fraction of the current carried by calcium by simultaneously measuring membrane current using whole-cell patch-clamp techniques and intracellular Ca^{2+} using the fluorescent indicator Fura-2. The Fura-2 response to free Ca^{2+} was calibrated individually for each cell. Two different calibration methods are compared: one uses voltage-activated Ca^{2+} channels, and the other uses the same ligand-gated channels that are being tested but in a pure Ca^{2+} solution. The two methods give quantitatively different results. The method using pure Ca^{2+} currents through ligand-gated channels calibrates the Fura-2 signal through the same influx pathway that generates the test response, thus controlling for the distribution of channels and ensuring a similar interaction between the incoming Ca^{2+} and Fura-2. In a physiologic solution containing 2.5 mM Ca^{2+} at a holding potential of −50 mV, the percentage of inward current carried by Ca^{2+} through NMDA receptors in hippocampal neurons is 12.4%. By comparison, in sympathetic neurons the percentage of current carried by Ca^{2+} through neuronal nAChRs is 4.7%, and through ATP-activated purinergic receptors it is 6.5%. These percentages can be used to estimate the amount of Ca^{2+} entry through these receptors during synaptic activation, but care must be exercised in considering the many subtypes of each receptor.

INTRODUCTION

N-methyl-D-aspartate (NMDA) receptors comprise a subtype of glutamate receptors that produce activity-dependent Ca^{2+} signals while contributing to synaptic transmission (MacDermott et al., 1986; Mayer and Westbrook, 1987). The high Ca^{2+} permeability of NMDA receptors underlies their participation in the initiation of long-term potentiation at some excitatory pathways (Madison et al., 1991; Bliss and Collingridge, 1993). Purinergic ATP receptors and neuronal nicotinic ACh receptors (nAChR) also are permeable to calcium (Fieber and Adams, 1991b; Vernino et al., 1992) and can participate in fast synaptic transmission within peripheral ganglia and the central nervous system (Edwards et al., 1992; Evans et al., 1992; Sargent, 1993). Although the relative Ca^{2+} permeability of these receptor channels has been clearly demonstrated from permeability ratios calculated from reversal potential measurements, the actual amount of Ca^{2+} influx is still open to question.

Recently, the percentage of current carried by Ca^{2+} through NMDA and nACh receptors was estimated based on a technique that calibrated the receptor-dependent Ca^{2+} signals using voltage-activated Ca^{2+} channels (VACCs) from different cells (Schneggenburger et al., 1993; Trouslard et al., 1993; Zhou and Neher, 1993). We have compared that technique with a newer approach (Vernino et al., 1994) that calibrates the receptor-dependent Ca^{2+} influx in physiologic solutions with pure Ca^{2+} signals through the same receptors in the same cells. Results from both approaches are compared. We estimate that in a physiologic solution containing 2.5 mM Ca^{2+} at a holding potential of −50 mV, the percentage of inward current carried by Ca^{2+} through NMDA receptors in hippocampal neurons is 12.4%, through neuronal nAChRs in sympathetic neurons is 4.7%, and through ATP-activated purinergic receptors in sympathetic neurons is 6.5%. The results can be used to estimate the amount of calcium that enters a cell from simple current measurements of the activation of these receptor channels.

MATERIALS AND METHODS

Hippocampal neurons were cultured using techniques described in detail previously (Zarei and Dani, 1994). The CA1 region of the hippocampus of neonatal Sprague-Dawley rats was incubated in 10 ml of Earle's balanced salt solution (Gibco BRL, Grand Island, NY) containing 200 units of papain for 30 min (Worthington Biochemical Corp., Freehold, NJ). The tissue was gently triturated in complete media composed of Minimum Essential Medium (Gibco), 5% heat-inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT), 50 units/ml penicillin, 50 μg/ml streptomycin, 20 mM glucose, 1 μl/ml serum extender (Collaborative Research, Bedford, MA), and 2.5 mg/ml trypsin inhibitor and bovine serum albumin (Sigma Chemical Co, St Louis, MO). Cells were plated on coverslips coated with collagen and poly-D-lysine and maintained in an incubator at 37°C, 5% CO_2. Media was changed every 3 days, and cells were treated for 2 days with 5 μM cytosine arabinofuranoside.

Sympathetic neurons were cultured according to the methods of Mathie et al. (1990). Superior cervical ganglia (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 units/ml collagenase and 150 units/ml DNAse (Worthington and Sigma) in Dulbecco's modified Eagles Medium (DMEM) (Gibco). The supernatant was removed, and the ganglia were incubated for 30 min in a solution of DMEM with 100 units/ml trypsin (Sigma) and a 50% dilution of the collagenase and DNAse solution. Ganglia were washed in DMEM

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containing 10% FBS to inactivate the trypsin and then triturated through a series of flamed Pasteur pipettes before being re-suspended in DMEM containing 10% FBS and 10–25 ng/ml NGF (Collaborative Research). Sympathetic neurons were plated onto coverslips coated with collagen and poly-D-lysine and were used over the course of 4–40 h. All of the SCG neurons lacked processes because adult sympathetic neurons are slow to grow neurites and the NGF concentration used here was only sufficient to promote survival.

Rat chromaffin cells were prepared according to the methods of Akaike et al. (1990). Postnatal week 3–4 Sprague-Dawley rats were sacrificed with halothane, the adrenal glands were quickly removed and washed with ice-cold, low Ca^{2+} buffer (in mM): 120 NaCl, 5.5 KCl, 1 $MgSO_4$, 0.85 NaH_2PO_4, 2.25 Na_2HPO_4, 0.1 $CaCl_2$, 10 glucose, 5 HEPES, pH 7.4. Adrenal medullae were isolated from the adrenal cortex and connective tissue, chopped into pieces, and rinsed with cold, low Ca^{2+} buffer before incubation for 15 min at 37°C in low Ca^{2+} buffer containing 0.3% collagenase (Worthington). Tissue fragments were transferred to warm Ca^{2+} buffer (2 mM $CaCl_2$) and washed several times. Cells were dissociated in low glucose DMEM with 1% bovine serum albumin and 2.5 mg/ml trypsin inhibitor (Sigma) by trituration through a series of fire-polished glass pipettes. Cells were suspended in low glucose media containing 10% heat-inactivated FBS (Gibco), 100 μg/ml each of penicillin (Sigma) and streptomycin (Gibco), and 10 ng/ml nerve growth factor (Sigma), and then plated directly onto coated coverslips on the bottoms of modified culture dishes. Cells were allowed to settle in an incubator at 37°C with 5% CO_2 and were used 4–36 h after plating.

Recording solutions were chosen to isolate ligand-gated receptor currents from other membrane conductances. Extracellular test solutions contained (in mM) 150 NaCl, 2.5 $CaCl_2$, and 10 HEPES. The extracellular pure Ca^{2+} solution contained 75 or 25 mM $CaCl_2$ and 10 mM HEPES. Solutions were adjusted to pH 7.4 with the hydroxide of the major cation and were osmotically adjusted to 0.3 osm with sucrose. Atropine and CNQX (both 2 μM; Sigma and Tocris Neuramin, Essex, UK) were added to block muscarinic and non-NMDA receptor activation during studies of nicotinic and NMDA receptor currents. All external test solutions additionally contained 250 nM TTX (Calbiochem, La Jolla, CA) to block voltage-dependent Na^+ channels. Fast agonist applications were made with large outflow tubes positioned in a row and mounted on a computer-controlled, high speed motorized drive (Vernino et al., 1992, 1994) (Newport Corp., Irvine, CA). The internal solution in the patch pipettes contained (in mM): 140 *N*-methyl-D-glucamine, 1 MgATP, 5 tetraethylammonium chloride (TEA), 1 Fura-2, and 20 HEPES. The solution was adjusted to pH 7.4 with methanesulfonic acid, which provided the main anion. These experimental conditions ensured that currents were only due to ion flux through the ligand-gated channel of interest, and the internal solution of impermeant ions ensured that receptor currents were all unidirectional, inward currents. Voltage-dependent and Ca^{2+}-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, whereas Ca^{2+}-activated Cl^- conductances were eliminated by replacing Cl^- with the impermeant anion methanesulfonate.

Simultaneous measurements of the intracellular calcium concentration and membrane current were made at the single-cell level using Fura-2 microfluorimetry and whole-cell patch-clamp techniques, as we described previously (Vernino et al., 1994). Currents were amplified and filtered using a Warner PC-501 amplifier with a 4-pole Bessel filter (Warner Inst. Corp., Hamden, CT). The cell-impermeant pentapotassium form of Fura-2 (Molecular Probes, Eugene, OR) was allowed to equilibrate within a cell before measurements of intracellular Ca^{2+} were made by exposing the specimen to rapidly alternating periods of 340 and 380 nm incident wavelength light and collecting Fura-2 fluorescence emission above 480 nm with a photomultiplier (Thorn EMI, Middlesex, U.K.). The free Ca^{2+} concentration was calculated using the equation of Grynkiewicz et al. (1985): $[Ca^{2+}] = K(R - R_{min})/(R_{max} - R)$, where R is the measured ratio of Fura-2 fluorescence measured every 17 ms. Calibrations of the microfluorimetry setup with Ca^{2+} standards gave values for K, R_{min}, and R_{max} of 2.0 μM, 0.3, and 4. A high intracellular concentration of 1 mM Fura-2 was used so that the indicator would overcome endogenous cytosolic buffers and more accurately reflect changes in free Ca^{2+} resulting from influx across the membrane (Neher and Augustine, 1992).

RESULTS

Simultaneous recordings of NMDA receptor current and the concentration of intracellular Ca^{2+} were used to determine the fraction of current carried by Ca^{2+}. Fig. 1 shows data from a whole-cell, voltage-clamped hippocampal neuron in the pure external Ca^{2+} solution (Fig. 1 *A*) and in an external solution containing 2.5 mM Ca^{2+} (Fig. 1 *B*). In pure external Ca^{2+}, the inward current is carried exclusively by Ca^{2+} ions, providing a calibration for this cell by associating a particular Fura-2 response with a known Ca^{2+} influx. By using the calibration for comparison, the magnitude of the Ca^{2+}

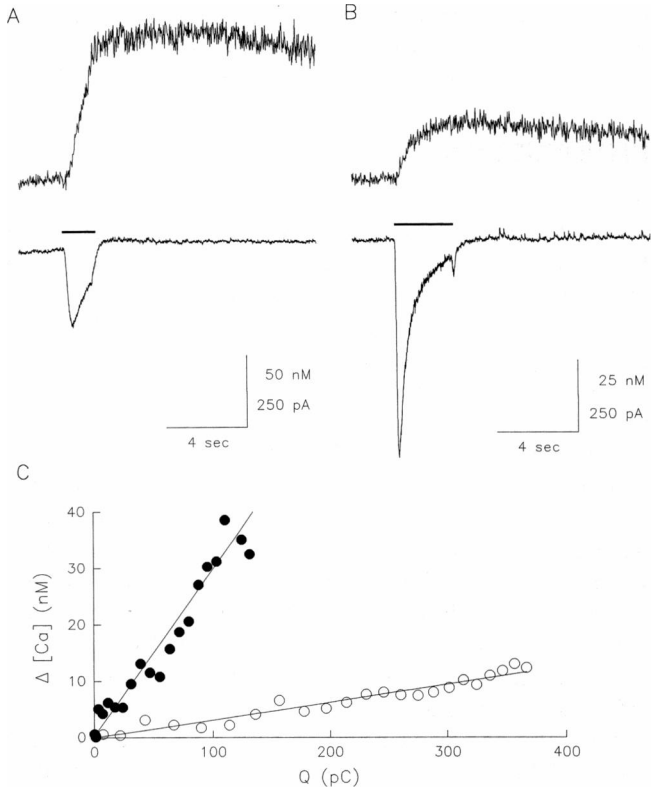

FIGURE 1 Calcium influx through NMDA receptors. Simultaneous recordings of NMDA receptor current (*lower* trace) and Fura-2 indication of intracellular Ca^{2+} concentration (*upper* trace) were made from a CA1 hippocampal neuron in an external solution of pure Ca^{2+} (*A*) and in a solution containing 2.5 mM Ca^{2+} (*B*). The neuron was perfused with an impermeant internal solution and voltage-clamped at −50 mV. Currents were activated by 20 or 80 μM NMDA (*A* and *B*, respectively) and 10 μM glycine. The solid horizontal bars above the currents indicate when the agonist was applied. Before and in between recordings, the cell was incubated in a Ca^{2+}-free solution containing 10 mM caffeine to deplete intracellular stores of Ca^{2+}, and the internal solution contained 1 μM thapsigargin to block uptake into stores. (*C*) The change in intracellular Ca^{2+} concentration (Δ[Ca]) is plotted against the cationic influx (Q) in pure Ca^{2+} (●) and in a physiologic solution of 2.5 mM Ca^{2+} (○). Each point represents the incremental increase in free Ca^{2+} and membrane current recorded every 17 ms during the first s of agonist application. Linear regression lines are drawn through the data points. The percentage of inward NMDA receptor current carried by Ca^{2+} for this neuron is 10.7%.

concentration change in a physiologic solution can be used to determine how much of the receptor current is carried by Ca^{2+}.

In Fig. 1 *C*, the change in intracellular Ca^{2+} (Δ[Ca]) during the agonist application is plotted against the integral of the membrane current, which is the amount of charge (Q) that has moved across the membrane through NMDA receptors. The abscissas of the plotted data points were obtained by integrating the current at successive times, and the ordinates were the Fura-2 indications of calcium concentration at those same times (see Vernino et al., 1994). In a solution of pure external Ca^{2+}, all of the incoming current is carried by Ca^{2+}. Therefore, a small charge influx (Q) produces a large change in the intracellular Ca^{2+} concentration (Δ[Ca]). In the external solution containing 2.5 mM Ca^{2+}, the slope of the Δ[Ca] vs. Q plot is smaller because only a fraction of the current is carried by Ca^{2+}. The fraction of current carried by Ca^{2+} in the physiologic solution was determined by dividing the slope of the Δ[Ca] vs. Q plots in pure Ca^{2+} into the slope obtained in the 2.5 mM Ca^{2+} solution. With the impermeant internal solution at a holding potential of -50 mV, the percentage of inward current carried by Ca^{2+} through NMDA receptors in hippocampal neurons is 12.4 $\pm$ 1.2% (n = 5).

The validity of this method has been extensively tested under many conditions (Vernino et al., 1994). The linear relationship between Δ[Ca] and Q during each agonist application (Figs. 1 *C* and 2 *C*) indicates that cellular processes, such as calcium buffering and release from intracellular stores, are not affecting the measurement. If Ca^{2+} release from intracellular stores had occurred during an agonist application, there would have been an upward curving of the Δ[Ca] $-$ Q relationship, which was not seen under our experimental conditions. We also controlled for Ca^{2+}-induced Ca^{2+} release (Llano et al., 1994) by bathing cells in a Ca^{2+}-free solution containing 10 mM caffeine to deplete Ca^{2+}-sensitive intracellular stores, and including 1–3 μM thapsigargin in the internal solution to inhibit Ca^{2+} uptake into stores (see Vernino et al., 1994).

The calibration method depicted in Fig. 1 relies on passing a pure Ca^{2+} current through the same NMDA receptors that are activated in the physiologic solution of 2.5 mM Ca^{2+}. Previously, Schneggenburger et al. (1993) used voltage-activated calcium currents measured in different cells to calibrate their NMDA receptor-dependent Fura-2 signal. We improved on this alternative method by obtaining the VACC calibration in the same cells that were used to record NMDA receptor currents. Because the Fura-2 indication of Ca^{2+} depends on the volume of the cell, calibrating each cell as it is used provides a more accurate indication of the fractional Ca^{2+} influx through the ligand-gated channels. An example of the two calibration methods obtained in one cell is shown in Fig. 2. A voltage-activated calcium current and associated Ca^{2+} transient is shown in Fig. 2 *A*, and the corresponding NMDA receptor current and calcium transient obtained in a pure external Ca^{2+} solution are shown in Fig. 2 *B*. The relationships between Δ[Ca] and Q for both sets of calibration data are plotted in Fig. 2 *C*, showing that the slope of the

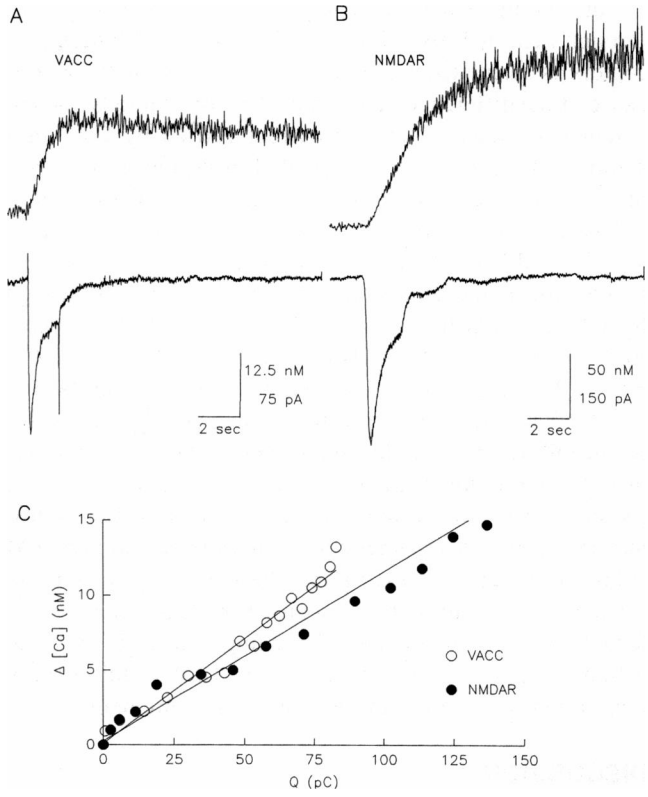

FIGURE 2 A comparison of the two different Fura-2 calibration methods described in the text. The change in intracellular Ca^{2+} concentration (Δ[Ca]) is plotted versus cationic influx (Q). The simultaneous recordings of current (*lower* traces) and Fura-2 indication of intracellular Ca^{2+} concentration (*upper* traces) are shown for VACCs obtained in a solution containing 2.5 mM Ca^{2+} (*A*) or for NMDA receptors obtained in a solution of pure Ca^{2+} (*B*). (*C*) The calibration line obtained using VACCs (○) is steeper than the calibration line obtained using NMDA receptor currents (●) from the same neuron. The calibration lines were obtained by drawing linear regressions through the data points.

calibration obtained with the VACC is steeper than the calibration slope obtained using NMDA receptors in the same cells. Therefore, the percentage of current carried by Ca^{2+} through NMDA receptors in a physiologic solution of 2.5 mM Ca^{2+} calculated from the VACC calibration is 9.7 $\pm$ 2.3% (n = 7), which is lower than the value of 12.4 $\pm$ 1.2% (n = 5) calculated using the pure Ca^{2+} calibration through NMDA receptors.

We found a consistent difference between the fractional Ca^{2+} influx calculated using each calibration method, independent of the cells or receptor channels studied. The VACC calibration slope in hippocampal neurons, sympathetic neurons, and chromaffin cells was always steeper than the calibration slope obtained using pure Ca^{2+} signals through NMDA, ATP, and neuronal nACh receptors in the same cells, respectively. The difference in calibration slopes was statistically significant for NMDA and ATP receptors (Wilcoxon signed rank test, $p \leq 0.05$), but the 13% difference in the calibration slopes for nAChRs from spherical chromaffin cells and acutely dissociated sympathetic neurons was not statistically significant.

Fig. 3 compares the percentage of current carried by Ca^{2+} through neuronal nAChRs, ATP receptors, and NMDA receptors as calculated using the two calibration methods. Because of the difference between calibration methods, the apparent percentage of inward current carried by Ca^{2+} in a solution of 2.5 mM Ca^{2+} calculated using the VACC calibration was consistently lower for neuronal nAChRs (3.9 ± 0.2%, n = 4), purinergic ATP receptors (5.1 ± 0.8%, n = 7), and NMDA receptors (9.7 ± 2.3%, n = 7) compared with values calculated using pure Ca^{2+} signals through ligand-gated nAChRs (4.6 ± 0.4%, n = 9), ATP receptors (6.5 ± 0.4%, n = 6), and NMDA receptors (12.4 ± 1.2%, n = 5). Because α7 homo-oligomeric nAChRs expressed in oocytes have been shown to have exceptionally high Ca^{2+} permeabilities, but are blocked by nanomolar concentrations of α-bungarotoxin (Séguéla et al., 1993), we tested for the presence of α-bungarotoxin-sensitive nAChRs. Both chromaffin cells and sympathetic neurons were bathed in 50 nM α-bungarotoxin for 40–60 min. The percentage of current carried by Ca^{2+} through the neuronal nAChRs was not affected by the treatment, indicating there is not an α-bungarotoxin-sensitive subtype of nAChR (in this affinity range) contributing to our percentage measurements.

DISCUSSION

These three ligand-gated receptor families exhibit functional diversity, particularly when cloned receptors are studied in expression systems (Kutsuwada et al., 1992; Sargent, 1993; Barnard et al., 1994). Under physiological conditions in a neuron, however, this diversity may be diminished, especially when considering a single functional property such as Ca^{2+} flux. For example, α-bungarotoxin-insensitive neuronal nAChRs in chromaffin cells, sympathetic neurons, and expressed in oocytes all have a permeability ratio of Ca^{2+} to Na^+ or Cs^+ near 1.3 (Fieber and Adams, 1991a; Vernino et al., 1992; Séguéla et al., 1993; Costa et al., 1994). Chromaffin cells and rat SCG neurons express a wide range of nicotinic receptor subunits (Criado et al., 1992; Mandelzys et al., 1994) but exhibit similar ganglionic-type nAChR currents (Akaike et al., 1990; Mathie et al., 1990). Furthermore, the percentage of current carried by Ca^{2+} is nearly the same in these two cell types: 4.4 ± 0.4% (n = 4) in rat chromaffin cells and 4.7 ± 0.3% (n = 5) in rat sympathetic neurons (Fig. 3). Therefore, the percentages of Ca^{2+} influx presented here can be used, with caution, to provide valuable estimates of the activity-dependent Ca^{2+} signals produced by these ligand-gated channels.

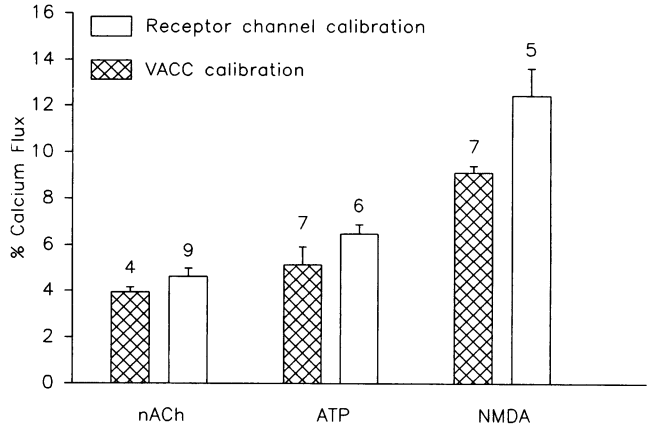

FIGURE 3 The percentage of inward current carried by Ca^{2+} in a physiologic solution of 2.5 mM Ca^{2+} at a holding potential of −50 mV is shown for neuronal nAChRs from adrenal chromaffin cells and sympathetic neurons, for ATP receptors from sympathetic neurons, and for NMDA receptors from CA1 hippocampal neurons. For the nAChRs, the data are pooled from sympathetic neurons (4.7 ± 0.3%, n = 5) and chromaffin cells (4.4 ± 0.4%, n = 4) because the results from the two cell types were essentially the same. These neuronal nAChR calcium measurements were insensitive to 50 nM α-bungarotoxin. The percentages are shown for calculations using calibrations obtained using VACCs (*hatched bars*) or using calibrations obtained in pure Ca^{2+} using the ligand-gated channels (*open bars*). The percentage presented for the calibration obtained using ligand-gated neuronal nAChRs is from Vernino et al. (1994); for ATP receptors, the percentage is from M. Rogers and J. A. Dani (in preparation). Those percentages are shown for comparison with the percentages obtained here with the VACC calibration method.

We have used two calibration methods to measure Ca^{2+} flux through ligand-gated receptor channels and find that they give quantitatively different answers. Such variations in Ca^{2+}-flux measurements, either between different calibration methods or among trials using the same method, could be attributed to differences in cells, culture conditions, recording solutions, or instrumentation. However, we have used identical culture and recording conditions and the same experimental equipment to reveal systematic differences between the two methods for calibrating the Ca^{2+} flux through receptor channels. The difference in the estimated Ca^{2+} flux based on the two calibration methods is small for receptors where the percentage of current carried by Ca^{2+} is small, but the difference becomes more significant for NMDA receptors, which have higher Ca^{2+} fluxes (Fig. 3). Our results with NMDA receptors using VACCs for the calibration are similar to those obtained by Schneggenburger et al. (1993), who estimated that in a solution of 1.8 mM Ca^{2+} 6.8% of the current is carried by Ca^{2+}, whereas we found 9.7% in 2.5 mM Ca^{2+} (both using the VACC calibration method).

Both methods for calibrating the Fura-2 signal rely on the assumption that the Fura-2 response during the calibration is the same as during the test. The calibration in pure external Ca^{2+} uses the same ligand-gated channels that are being tested and ensures that the distribution of Ca^{2+} entry into the cell is the same during the calibration and test. VACCs are distributed differently over the surface of neurons compared with ligand-gated channels (Hockberger et al., 1989; Benke et al., 1993). Thus, Ca^{2+} influx through VACCs may activate Fura-2 with different kinetics and spatial distribution, leading to different amounts of local Fura-2 saturation. We would expect this effect to be reduced in spherical chromaffin cells and acutely dissociated sympathetic neurons, where the difference in fractional Ca^{2+} current estimated by the two methods is smaller than in hippocampal neurons (Fig. 3), where the varied distribution of VACCs and NMDA receptors

could be more pronounced (Benke et al., 1993). These differences in Fura-2 buffering and kinetics are avoided by using the same channels for the calibration and the test, as demonstrated by the finding that the calibration method using ligand-gated channels is very insensitive to the rate of Ca^{2+} influx (Vernino et al., 1994). For this reason, we favor the results obtained using the ligand-gated channels for both the calibration and the test.

Because the internal solution used here does not contain permeant cations, the data in Fig. 3 give the percentage of one-way inward receptor current carried by Ca^{2+}. At a holding potential of −50 mV with a physiologic internal solution, there is a net inward current composed of a unidirectional inward component and a smaller unidirectional outward current. It is possible to calculate the fractional current carried by Ca^{2+} in a physiologic solution of 2.5 mM Ca^{2+} at −50 mV from these data, as was determined by Vernino et al. (1994). The percentage of net current carried by Ca^{2+} is 4.9% for nAChRs, 7.4% for ATP receptors, and 14.3% for NMDA receptors. There is a voltage dependence to this Ca^{2+} percentage, but far from the reversal potential of the current (in the range from −30 to −80 mV) the percentages are rather constant. Therefore, based on the unidirectional and net current Ca^{2+} fluxes presented here, it is possible to use current measurements to estimate how much Ca^{2+} has entered a neuron through these particular ligand-gated channels. Vernino et al. (1994) performed experiments with nAChRs with and without 1 mM Mg^{2+} added to the external solution and found no significant difference in the percentage of current carried by Ca^{2+}. Therefore, physiological concentrations of Mg^{2+} do not have a potent effect on the percentage of current carried by Ca^{2+}. Higher concentrations of Mg^{2+} could influence the permeation of Na^+ more than the permeation of Ca^{2+} because the divalent ions have higher affinities for sites along the permeation pathways of the nAChR pore (Decker and Dani, 1990) and the NMDA receptor pore (Jahr and Stevens, 1993; Zarei and Dani, 1994).

NMDA, ATP, and nicotinic ACh receptors underlie excitatory synaptic transmission at central and peripheral synapses. Calcium influx through these receptors can activate Ca^{2+}-dependent conductances capable of modulating neuronal excitability (Mulle et al., 1992; Tokimasa and North, 1984; Vernino et al., 1992; Zorumski et al., 1989) and can activate various Ca^{2+}-dependent enzymes to produce feedback regulation of the same receptors (Lieberman and Mody, 1994; Miles et al., 1994; Raymond et al., 1993). There is compelling evidence for a presynaptic localization of neuronal nAChRs (Sargent, 1993), where the Ca^{2+} influx through these receptors could modulate neurotransmitter release. Therefore, the measurements presented here of the actual magnitude of the Ca^{2+} influx through ligand-gated nicotinic, purinergic, and glutamatergic receptors provides a quantitative estimate of the Ca^{2+} signals functionally coupled to excitatory synaptic transmission in the peripheral and central nervous system.

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REFERENCES

Akaike, A., Y. Mine, M. Sasa, and S. Takaori. 1990. Voltage and current clamp studies of muscarinic and nicotinic excitation of the rat adrenal chromaffin cells. *J. Pharmacol. Exp. Ther.* 255:333–339.

Barnard, E. A., G. Burnstock, and T. E. Webb. 1994. G-protein coupled receptors for ATP and other nucleotides: a new receptor family. *Trends Pharmacol. Sci.* 15:67–70.

Benke, T. A., O. T. Jones, G. L. Collingridge, and K. J. Angelides. 1993. N-methyl-D-aspartate receptors are clustered and immobilized on dendrites of living cortical neurons. *Proc. Natl. Acad. Sci. USA.* 90: 7819–7823.

Bliss, T. V. P., and G. L. Collingridge. 1993. A synaptic model of memory: long-term potentiation in the hippocampus. *Nature.* 361:31–39.

Costa, A. C. S., J. W. Patrick, and J. A. Dani. 1994. Improved technique for studying ion channels expressed in *Xenopus* oocytes, including fast superfusion. *Biophys. J.* 67:395–401.

Criado, M., L. Alamo, and A. Navarro. 1992. Primary structure of an agonist binding subunit of the nicotinic acetylcholine receptor from bovine adrenal chromaffin cells. *Neurochem. Res.* 17:281–287.

Decker, E. R., and J. A. Dani. 1990. Calcium permeability of the nicotinic acetylcholine receptor: the single-channel calcium influx is significant. *J. Neurosci.* 10:3413–3420.

Edwards, F. A., A. J. Gibb, and D. Colquhoun. 1992. ATP receptor-mediated synaptic current in the central nervous system. *Nature.* 359:144–147.

Evans, R. J., V. Derkach, and A. Surprenant. 1992. ATP mediates fast synaptic transmission in mammalian neurons. *Nature.* 357:503–505.

Fieber, L. A., and D. J. Adams. 1991a. Acetylcholine-evoked currents in cultured neurones dissociated from rat parasympathetic cardiac ganglia. *J. Physiol.* 434:215–237.

Fieber, L. A., and D. J. Adams. 1991b. Adenosine triphosphate-evoked currents in cultured neurones dissociated from rat parasympathetic cardiac ganglia. *J. Physiol.* 434:239–256.

Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

Hockberger, P. E., H. Y. Tseng, and J. A. Connor. 1989. Fura-2 measurements of cultured rat Purkinje neurons show dendritic localization of Ca^{2+} influx. *J. Neurosci.* 9:2272–2284.

Jahr, C. E., and C. F. Stevens. 1993. Calcium permeability of the N-methyl-D-aspartate receptor channel in hippocampal neurons in culture. *Proc. Natl. Acad. Sci. USA.* 90:11573–11577.

Kutsuwada, T., N. Kashiwabuchi, H. Mori, K. Sakimura, E. Kushiya, K. Araki, H. Meguro, H. Masaki, T. Kumanishi, M. Arakawa, and M. Mishina. 1992. Molecular diversity of the NMDA receptor channel. *Nature.* 358:36–41.

Lieberman, D. N., and I. Mody. 1994. Regulation of NMDA channel function by endogenous Ca^{2+}-dependent phosphatases. *Nature.* 369:235–239.

Llano, I., R. DiPolo, and A. Marty. 1994. Calcium-induced calcium release in cerebellar Purkinje cells. *Neuron.* 12:663–673.

Mandelzys, A., B. Pie, E. S. Deneris, and E. Cooper. 1994. The developmental increase in ACh receptor densities on rat sympathetic neurons correlates with changes in nicotinic ACh receptor α-subunit gene expression and occurs independent of innervation. *J. Neurosci.* 14: 2357–2364.

Mathie, A., D. Colquhoun, and S. C. Cull-Candy. 1990. Rectification of currents activated by nicotinic acetylcholine receptors in rat sympathetic ganglion neurones. *J. Physiol.* 427:625–655.

MacDermott, A. B., M. L. Mayer, G. L. Westbrook, S. J. Smith, and J. L. Barker. 1986. NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones. *Nature.* 321:519–522.

Madison, D. V., R. C. Malenka, and R. A. Nicoll. 1991. Mechanisms underlying long-term potentiation of synaptic transmission. *Annu. Rev. Neurosci.* 14:379–397.

Mayer, M. L., and G. L. Westbrook. 1987. Permeation and block of N-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurones. *J. Physiol.* 394:501–527.

Miles, K., S. S. M. Audigier, P. Greengard, and R. L. Huganir. 1994. Autoregulation of phosphorylation of the nicotinic acetylcholine receptor. *J. Neurosci.* 14:3271–3279.

Mulle, C., D. Choquet, H. Korn, and J. P. Changeux. 1992. Calcium influx through nicotinic receptor in rat central neurons: its relevance to cellular regulation. *Neuron.* 8:135–143.

Neher, E., and G. J. Augustine. 1992. Calcium gradients and buffers in bovine chromaffin cells. *J. Physiol.* 450:273–301.

Raymond, L. A., C. D. Blackstone, and R. L. Huganir. 1993. Phosphorylation of amino acid neurotransmitter receptors in synaptic plasticity. *Trends Neurosci.* 16:147–153.

Sargent, P. 1993. The diversity of neuronal nicotinic acetylcholine receptors. *Annu. Rev. Neurosci.* 16:403–443.

Schneggenburger, R., Z. Zhou, A. Konnerth, and E. Neher. 1993. Fractional contribution of calcium to the cation current through glutamate receptor channels. *Neuron.* 11:133–143.

Séguéla, P., J. Wadiche, K. Dineley-Miller, J. A. Dani, and J. W. Patrick. 1993. Molecular cloning, functional expression and distribution of rat brain $\alpha 7$: a nicotinic cation channel highly permeable to calcium. *J. Neurosci.* 13:596–604.

Tokimasa, T., and R. A. North. 1984. Calcium entry through acetylcholine channels can activate potassium conductance in bullfrog sympathetic neurons. *Brain Res.* 295:364–367.

Trouslard, J., S. J. Marsh, and D. A. Brown. 1993. Calcium entry through nicotinic receptor channels and calcium channels in cultured rat superior cervical ganglion cells. *J. Physiol.* 468:53–71.

Vernino, S., M. Amador, C. W. Luetje, J. Patrick, and J. A. Dani. 1992. Calcium modulation and high calcium permeability of neuronal nicotinic acetylcholine receptors. *Neuron.* 8:127–135.

Vernino, S., M. Rogers, K. A. Radcliffe, and J. A. Dani. 1994. Quantitative measurement of calcium flux through muscle and neuronal nicotinic acetylcholine receptors. *J. Neurosci.* 14:5514–5524.

Zarei, M. M., and J. A. Dani. 1994. Ionic permeability characteristics of the N-methyl-D-aspartate receptor. *J. Gen. Physiol.* 103:231–248.

Zhou, Z., and E. Neher. 1993. Calcium permeability of nicotinic acetylcholine receptor channels in bovine adrenal chromaffin cells. *Pflügers Arch.* 425:511–517.

Zorumski, C. F., L. L. Thio, G. D. Clark, and D. B. Clifford. 1989. Calcium influx through N-methyl-D-aspartate channels activates a potassium current in postnatal rat hippocampal neurons. *Neurosci. Lett.* 99:293–299.