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Flow Injection Microscopy for the Study of Intracellular Calcium Mobilization by Muscarinic Agonists[1]

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The study of cellular response to chemical agonists is essential in understanding the complex functions mediated by cell surface receptors. Flow injection microscopy has been used with the CHO-M1-WT3 cell line and the fluorescent Ca^{2+} indicator Fura-2-AM to monitor mobilization of internal Ca^{2+}. Repeated stimulation of cells mounted in an inverted radial flow chamber allows the direct comparison of relative intracellular Ca^{2+} mobilization with respect to agonist dose. The process of determining dose–response relationships is simplified since an entire dose–response curve can be constructed from a distinct set of cells. Use of flow injection lends precision to the application and removal of agonists while allowing cellular activity to be monitored throughout the stimulation and recovery processes. In this work, dose–response curves have been constructed for the muscarinic agonists carbachol, acetylcholine, and pilocarpine resulting in EC_{50} values of 1.7 μM, 56 nM, and 6.8 μM, respectively.

Muscarinic receptors have been the topic of numerous pharmacological studies due to their fundamental significance in cellular function and the potential for their importance in the treatment of Alzheimer's and other disease (1–4). Muscarinic receptors and their agonists play an important role in cellular regulation of inositol 1,4,5-trisphosphate from phosphatidylinositol 4,5-bisphosphate hydrolysis and the consequent release of Ca^{2+} from internal stores (5, 6). One result of their stimulation is an increase in intracellular free Ca^{2+} which, because of the complexity of the Ca^{2+} release pathway, is extremely sensitive to agonist type and concentration (7, 8).

Several methods have been used in the study of receptor-mediated calcium response. One of the most common experimental methods involves permeabilization of cellular membranes. Cells are incubated with $^{45}Ca^{2+}$, electrically permeabilized, and stimulated with agonist and the release of the radioactive species is monitored (9). This method, in addition to requiring the use of radiochemicals, assumes that cellular function after extensive preparation will be the same as for viable cells. The ultimate response mediated by a receptor is not determined simply by its binding of a ligand but is the end result of a pathway dependent on receptor abundance, availability of G-proteins, and numerous other factors including endocytosis of receptor sites (7, 10). Assays which rely on cellular permeabilization may be hampered by inaccurately accounting for these factors. Flow cytometry has been used to observe intracellular calcium mobilization in intact cells (11). However, this method can often lack the reproducible timing which is required for kinetic studies and does not allow for cellular imaging. Moreover, the use of flow cytometry is limited to a few cell lines which can be handled as suspensions and are robust to substantial flow-induced stresses.

Fluorescence microscopy has proven to be a powerful means of detecting receptor activation using the fluorescent Ca^{2+} indicator Fura-2-acetoxymethyl ester (Fura-2-AM)[2] (5, 6, 12, 13). To date, batch methods which necessitate the collection of individual data points on separate cells or sets of cells have been used. The application of an agonist to cells is typically carried out manually, introducing undesirable variation between experiments. Lack of precision in the dosing rate and contact concentration of a reagent addition

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[2] Abbreviations used: Fura-2-AM, Fura-2-acetoxymethyl ester; FIM, flow injection microscopy; HBSS, Hank's balanced salt solution; Fura-2-FA, Fura-2-pentapotassium salt.

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engenders a lack of control with respect to the duration of exposure.

Flow injection microscopy (FIM) has been introduced as a means for monitoring cellular response in adherent cells using a well-characterized and reproducible flow regime (14). This communication introduces a method which allows precise and automated screening of agonists for the rapid construction of dose–response curves. The FIM method can not only monitor cellular response continuously, but also the response can be directly related to the application of agonist by overlaying a tracer curve. The tracer curve, a flow profile, reveals the exact concentration of agonist in contact with the cells at each time point. The use of a FIM flow regime was found to be a significant improvement to previous manual experimentation through increased reproducibility, speed, and ease with which dose–response curves can be constructed. Also, experimental accuracy is improved since the covariance of cellular response changes to agonist dose changes can be studied for the first time with exceptional temporal resolution. Repeated stimulation of distinct sets of cells can be easily performed, and it is shown that separate cell populations respond to agonist stimulation in a consistent and reproducible manner.

MATERIALS AND METHODS

Cell Culture

Chinese hamster ovary cells transfected with the M1 muscarinic receptor (CHO-M1-WT3) were cultured in Ham's F-12 medium with 10% heat-inactivated fetal bovine serum and 50 μg/liter geneticin. The above reagents were supplied by Gibco BRL (Gaithersburg, MD). Cells were approximately 80% confluent when passaged. Trypsin (0.25%) was used to release cells from culture plates during passaging. Passage numbers 37–60 were used. Cellular activity was consistent within a given passage number, but overall activity was found to be reduced with successive passage numbers over 60.

Cell Culture Preparation

CHO-M1-WT3 cells were plated into two-sided Lab Tek chambers with a No. 1 borosilicate coverslip bottom (Nunc chambers; Nunc, Naperville, IL) for experimentation. Bovine plasma fibronectin, 1 mg/liter DI H_2O (Life Technologies Inc., Grand Island, NY) was applied to the inner surface of Nunc chambers. To each chamber, 1 cm^3 fibronectin was added. After 20 min, fibronectin was aspirated and chambers were rinsed twice with 3 cm^3 sterile irrigation water. Cell suspension (1 cm^3) of the desired concentration was pipetted into each coverslip chamber. After 1–3 days incubation (37°C, 5% CO_2), growth medium was aspirated from the chambered coverslip. The cells were washed with 1 cm^3 Hank's balanced salt solution (HBSS) three times. A 1-cm^3 aliquot of 1 μM Fura-2-AM (Molecular Probes, Eugene, OR) solution in HBSS was added to the cells. Cells were permeated with Fura-2-AM for 20 min at room temperature. Because Fura-2-AM is light sensitive, the chambers were kept shrouded during permeation. Fura-2-AM solution was aspirated, and cells were washed three times with 1 cm^3 HBSS. Cells were then covered with 2 cm^3 HBSS.

Unless otherwise noted, chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) with the exception of pilocarpine (Pilo) which was obtained from RBI (Natick, MA).

Instrumentation

The epifluorescent microscope and fluidics used in this work have been previously described (14) (Fig. 1). Briefly, a Zeiss Axiovert 100 inverted microscope (Carl Zeiss, Oberkochen, Germany) was used with a 40×/1.3 NA Fluar oil immersion objective. The signal was detected by a Photon Technology International Model SE-510 photometer head and amplified by a Model 710 photomultiplier tube (PMT). Flow injection fluidics consisted of a portable FIAlab-3000 (Alitea Instruments, Medina, WA) controlled by FIAlab for Windows (Alitea Instruments) on a 486DX2 66 MHz laptop computer (Austin Direct, Austin, TX). Data were collected on a Dell Pentium 133 MHz desktop computer using FELIX software (Photon Technology International), and analyzed using FELIX and Microsoft EXCEL software.

A radial flow chamber was constructed from a block of Plexiglas (76 × 56 × 18 mm) with support screws at the corners. An inlet Plexiglas tube was held into the block by a set screw. Within the inlet Plexiglas tube was precut stainless-steel tubing (0.030 in. i.d. × $\frac{1}{16}$ in. o.d. × 0.5 m; Upchurch Scientific, Oak Harbor, WA) which connected to the multiport syringe pump. The chamber outlet was formed by inserting an 18-gauge syringe needle into the block at the level corresponding to 1.5 cm^3 fluid in the chamber. The bottom of the chamber was formed by the Nunc chambered borosilicate glass coverslip. The gap between the inlet and the chamber bottom was set at 0.16 mm.

The Nunc chamber and the Plexiglas block with attenuate hardware were held stationary by an aluminum holder which fit into the microscope stage. The holder supported the Nunc chamber over the microscope objective. The support screws of the Plexiglas block fit into the holder so that the inlet was held in position in the center of the coverslip chamber.

A carousel-style autosampler was constructed in-house and was controlled by software written in MS-DOS to run independently of the FIM system on an

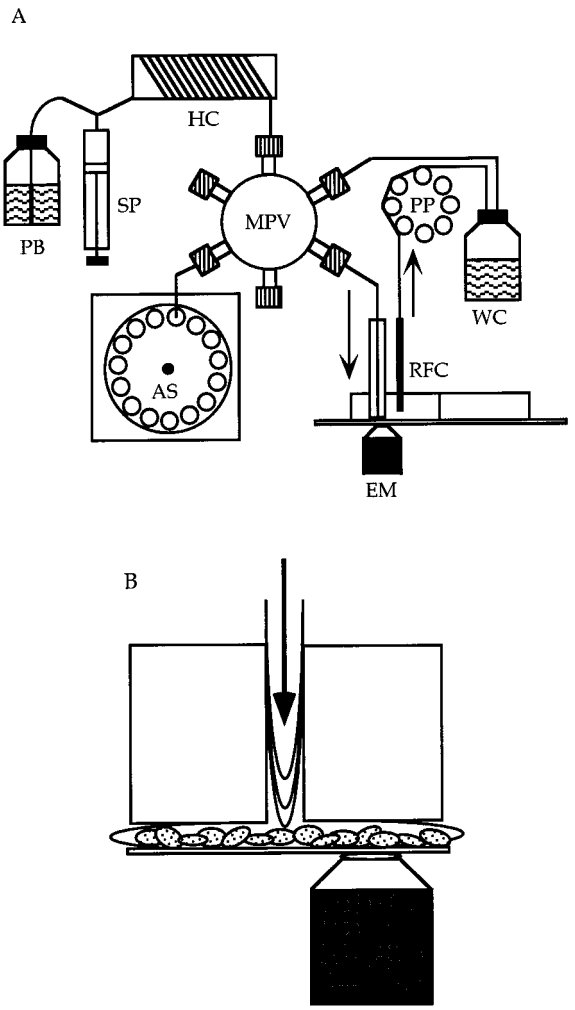

FIG. 1. Experimental setup for flow injection microscopy. (A) PB, perfusion buffer; SP, syringe pump; HC, holding coil; MPV, multiport valve; AS, autosampler; RFC, radial flow chamber; EM, epifluorescent microscope; PP, peristaltic pump; WC, waste collection. (B) Detail of radial flow chamber.

Austin 486 laptop computer. Interface of the autosampler with the FIA system allowed the introduction of up to 15 samples using only two ports on the system's Cavro Multi-Port-Valve. In addition to facilitating the construction of more extensive dose–response curves, sample switching was simplified since a new sample could be added to the carousel at any time without a requisite rinsing of the FIA system.

CHO-M1-WT3 cells plated into Nunc chambered coverslips were positioned in the RFC apparatus with 2 cm^3 buffer covering the cells. Fluorescence excitation data acquisition was begun, and a baseline level was acquired for 30 s. Data were collected at 5 Hz.

Dose–Response Curve Injection Sequence

Prior to data collection, up to five standard injections of 10 μM Cch were applied to the cells. This procedure served to prime the system. That is, any cells which were not well attached were flushed, responsiveness of cells and absence of bubbles were verified, and the operating fluid level in the RFC was established. Each experiment consisted of 28 to 32 agonist doses followed by 9 injections of a nonagonist, cell impermeant dye [Fura-2-pentapotassium salt (Fura-2-FA)]. Each dose consisted of a 50-μl bolus of agonist followed by 1950 μl HBSS which served to remove the agonist from cellular contact. Doses within a sequence were applied in ascending order. Figure 2 gives raw data collected from an injection sequence of acetylcholine. A complete assay included the entire injection sequence repeated in triplicate. Carbachol standards as described below were injected after every fourth or eighth experimental dose. The injections of Fura-2-FA were used to calculate applied concentration levels as outlined below.

Determination of Agonist Concentration

An obstacle anticipated with this method was variability in the dose of agonist applied to the specific set of cells being monitored. Immobilization of the RFC inlet has eliminated a large degree of variability within and between experiments due to movement of the inlet while still allowing the targeting of specific cell populations. However, some variability may still be caused by slight differences in focus and positioning of the chambered coverslip. Therefore, several tracer injections of Fura-2-FA are used to calculate the actual concentration of agonist applied to cells. This method also takes into account dilution due to parabolic flow within the FIA system. At the end of each agonist series, three square impulse injections of 800 μl of 0.4 mM Fura-2-FA followed by three square impulse injections of 800 μl of 1 μM Fura-2-FA were applied. These were followed by five 50-μl injections also of 1 μM Fura-FA. All Fura-2-FA peaks were background subtracted, and each set of injections was averaged. A linear regression of the square impulse sets was carried out. The average of the 50 μl injections was then fit to the regressed data to give the dilution factor. Each agonist sample concentration was then multiplied by this dilution factor to calculate the applied concentration of agonist. This technique is similar to the standard FIA method for the calculation of maximum dilution (D_{max}) in a flow system (15).

Data Analysis

Regression analysis was performed on the background-subtracted standard injections (10 μM carbachol) from each experiment (Microsoft EXCEL 5.0). The regression data were then used to calculate a standardized peak value at each time point for the duration of the experiment. Background-subtracted

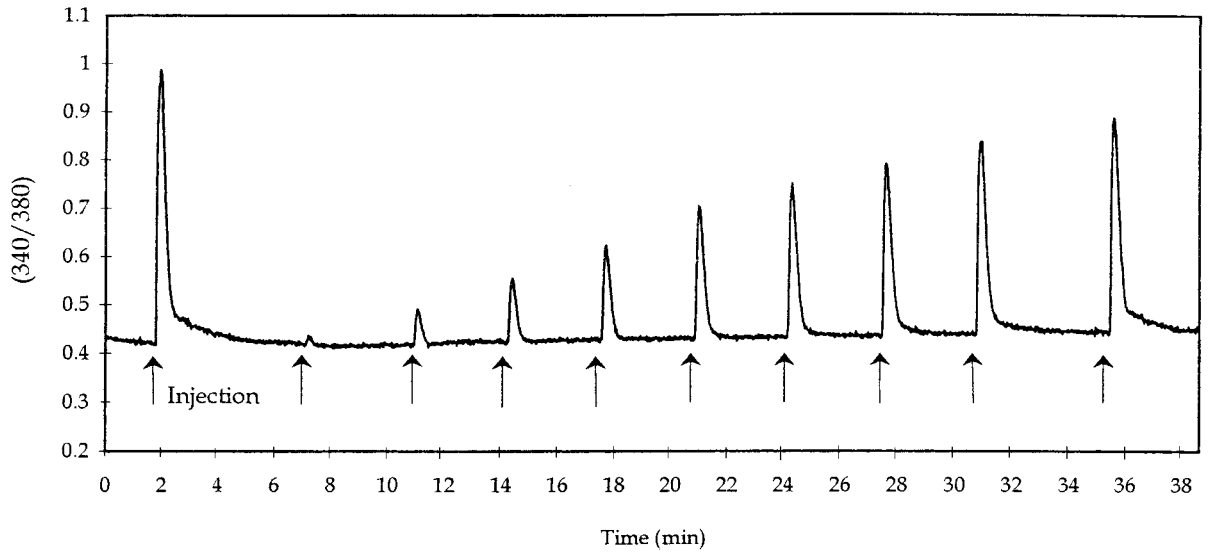

FIG. 2. Experimental dose sequence for acetylcholine. Peaks 1 and 10 are standard injections of 10 μM carbachol. Acetylcholine doses of 0.10, 0.15, 0.20, 0.30, 0.40, 0.60, 0.80, and 1.0 μM comprise the agonist injection series, peaks 2–9. Arrows indicate time of injection.

signal peaks were taken as a fraction of the calculated standard value at the time point of signal response. The three corrected peak values for a given agonist concentration were then averaged.

RESULTS AND DISCUSSION

Flow injection microscopy has proven to be a reliable and effective tool for the determination of dose–response relationships elicited by muscarinic receptor–agonist interaction.

Multiple Stimulation

Traditionally, individual sets of adherent cells have not been used for multiple stimulation experiments because, due to desensitization of receptor sites, response intensity decreases with each consecutive stimulation. Desensitization is here defined as a decrease in signaling intensity due to short-term (seconds to minutes) agonist exposure and is mediated by uncoupling of receptors to signal-transducing G-proteins (7). The maximal number of stimulations reported on a single set of cells was 3 (6, 14). In the interest of determining reproducibility of cellular response to multiple stimulations, an experiment was conducted in which a single set of CHO-M1-WT3 cells was stimulated 60 times with 50-μl injections of 100 μM carbachol. Although sensitivity decreased under such extreme conditions, the cells remained viable and responsive throughout the 266-min experiment. A linear decrease in signal intensity was observed throughout the experiment. Application of the linear regression correction outlined above resulted in a correlation coefficient of 0.9903 for the background-corrected peaks.

An experiment was conducted to investigate the effect of flow rate on response intensity. The rate of decrease of the response at a flow rate of 200 μl s^{-1} approached that for a flow rate of 50 μl s^{-1}, possibly indicating that desensitization involves only a small fraction of the total number of receptors with each stimulation (Fig. 3). Because the rate of signal decrease was constant within a given experiment, linear regression of standards was used to correct the data. The generally weaker intensity of response at the faster flow rate was due to increased dilution of the agonist bolus. As seen in the relationship

$$D_f = \frac{2\pi^{3/2} r^2 D_{ax}^{1/2} V_r^{1/2}}{V_s Q^{1/2}},$$

where the dilution factor D_f is related to the tube radius r, the axial dispersion coefficient D_{ax}, reactor and sample volumes, V_r and V_s, respectively, and the flow rate Q, dilution is inversely related to the square root of flow rate (15). An increase in flow rate from 50 to 200 μl s^{-1} increases the dilution factor by 50%.

Dose–Response Curves

Dose–response curves constructed from distinct injection sequences compare well. Figure 4 represents two such curves wherein separate cells from a single passage were used, and dilution factors and curve fits were determined independently. The results shown

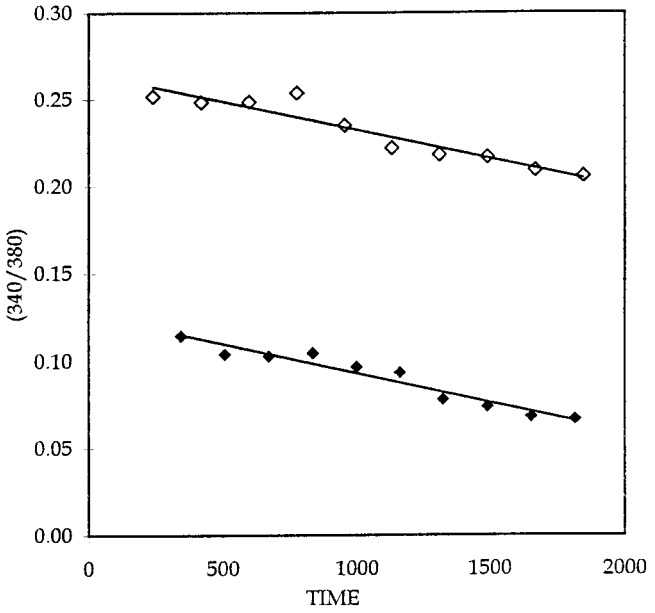

FIG. 3. Decrease in response intensity with multiple stimulations. (◇) Flow rate, 50 μl s^{-1}; slope, -3.28×10^{-5}; (◆) flow rate, 200 μl s^{-1}; slope, -3.37×10^{-5}.

differ by less than 0.5%. The data were fit by iteration to the equation

$$R = \frac{-R_{max}}{1 + (C/EC_{50})^{b}} + R_{max},$$

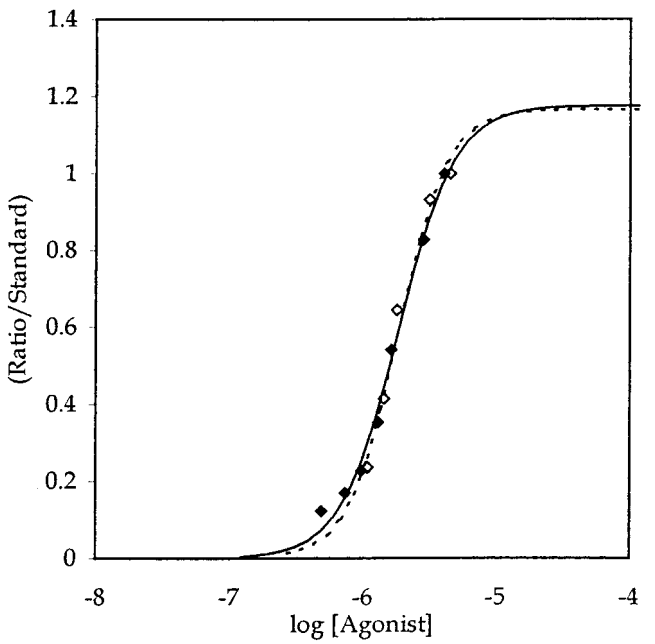

FIG. 4. Reproducibility of the method. Independently constructed dose–response curves for carbachol. Error bars have been omitted for clarity. Experiment 1: ◇, data; (- - -), model; EC_{50} = 1.74 μM. Experiment 2: ◆, data; (—), model; EC_{50} = 1.73 μM.

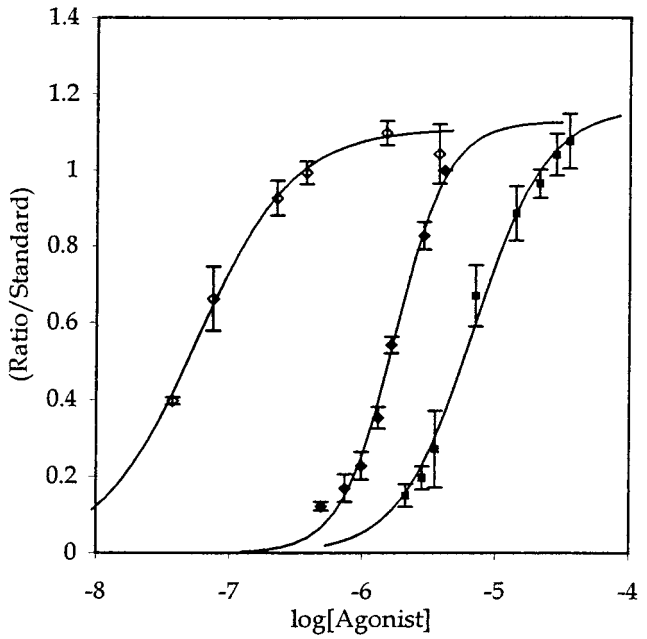

FIG. 5. Dose–response curves for (◇) acetylcholine, (◆) carbachol, and (■) pilocarpine.

where concentration C produces response R for a given agonist, R_{max} is the response at saturation, EC_{50} is the concentration eliciting half-maximal response, and b is a constant related to the slope of the curve (16). The correlation coefficients for the two curves are 0.9903 and 0.9894, respectively.

Figure 5 displays representative dose–response curves constructed during this study. Each curve was constructed from a single, complete dose sequence. Sequences consisted of three repetitions of each dose. Data points represent response as percentage relative to carbachol standards (10 μM) and are means ± SE for the three repetitions within the experimental sequence. The EC_{50} value obtained from these curves for carbachol (EC_{50} = 1.7 μM) compares well to the literature value given by Baxter *et al.* (17) for a related manual technique (EC_{50} = 4.4 μM). An EC_{50} value of 6.8 μM was obtained for pilocarpine. This is similar to the value supplied by Gurwitz *et al.* (18) using an arachidonic acid release assay. No literature value was available with which to compare the EC_{50} value obtained for acetylcholine (56 nM).

The utility of standard regression was emphasized in the analysis of acetylcholine response data. Unlike the other agonists tested (including oxotremorine and arecoline, not shown), moderate to high doses of acetylcholine, represented in Fig. 5, affected an exponential decrease in standard response, whereas low doses (Fig. 2) produced a linear decrease. Analysis of standards allowed identification of and correction for this trend. It is suggested that the mechanism of desensi-

tization due to stimulation with acetylcholine at high doses is significantly different from that brought on by carbachol stimulation at doses evoking a similar response.

The main result of this work is a validation of FIM as a useful method for the study of biochemical responses to extracellular stimuli. This technique lends several advantages over those commonly in use. Repeated stimulation of a single set of cells allows direct comparison of biological response in real time while eliminating biovariability as a factor in comparison of independently collected data. Rates of response and recovery of Ca^{2+} mobilization can be monitored, and signal profile and behavior can be related to agonist type. This may ultimately prove useful in separating multiple signal transduction pathways coupled to the receptor.

Flow injection and the inverted radial flow chamber simultaneously provide consistency and precision in fluid handling with flexibility in setting and monitoring target populations of cells. The conditions under which the cells are stimulated can be easily characterized and maintained throughout and between experiments, and the agonist concentration in contact with the cells at any time can be determined. The relatively rapid flow rates possible with this technique allow a discrete pulse of agonist to be applied and immediately removed thus enabling accurate determination of latency behaviors previously unavailable.

The assay presented here allows the construction of a complete dose–response curve in less than 2 h. Application of an agonist standard at various points within the injection sequence facilitates correction due to receptor desensitization during the assay. Furthermore, determination of approximate concentration ranges may be facilitated by rapid screening of several agonists within a brief period of time.

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REFERENCES

1. Loudon, J. M., Bromidge, S. M., Brown, F., Clark, M. S., Hatcher, J. P., Hawkins, J., Riley, G. J., Noy, G., and Orlek, B. S. (1997) *J. Pharmacol. Exp. Ther.* **283,** 1059–1068.
2. DeLapp, N., Wu, S., Belagaje, R., Johnstone, E., Little, S., Shannon, H., Bymaster, F., Calligaro, D., Mitch, C., Whitesitt, C., Ward, J., Sheardown, M., Fink-Jensen, A., Jeppesen, L., Thomsen, C., and Sauerberg, P. (1998) *Biochem. Biophys. Res. Commun.* **244,** 156–160.
3. Fisher, A., Brandeis, R., Chapman, S., Pittel, Z., and Michaelson, D. M. (1998) *J. Neurochem.* **70,** 1991–1997.
4. Christopoulos, A., Pierce, T. L., Sorman, J. L., and El-Fakahany, E. E. (1998) *Mol. Pharmacol.* **53,** 1120–1130.
5. Mathes, C., Wang, S. S-H., Vargas, H. M., and Thompson, S. H. (1992) *Brain Res.* **585,** 307–310.
6. Wang, S. S-H., and Thompson, S. H. (1994) *Cell Calcium* **15,** 483–496.
7. Bohm, S. K., Grady, E., and Bunnett, N. W. (1997) *Biochem. J.* **322,** 1–18.
8. BraunerOsborne, H., Ebert, B., Brann, M. R., Falch, E., and KrogsgaardLarsen, P. (1996) *Eur. J. Pharmacol.* **313,** 145–150.
9. Wojciekiewicz, J. H., Safranay, S. T., Chaliss, R. A. J., Strupish, J., and Nahorski, S. R. (1990) *Biochemistry* **272,** 269–272.
10. Szekeres, P. G., Koenig, J. A., and Edwardson, J. M. (1998) *Mol. Pharmacol.* **53,** 759–765.
11. Jonas, L., Fulda, G., Damm, I., Nebe, B., and Rychly, J. (1995) *Acta Histochem.* **97,** 81–88.
12. Tojyo, Y., Tanimura, A., and Matsumoto, Y. (1998) *Life Sci.* **62,** 1635–1639.
13. Tojyo, Y., Tanimura, A., and Matsumoto, Y. (1997) *Cell Calcium* **22,** 455–462.
14. Ruzicka, J., Baxter, P. J., Thastrup, O., and Scudder, K. (1996) *Analyst* **121,** 945–950.
15. Ruzicka, J., and Hansen, E. H. (1988) Flow Injection Analysis, 2nd ed., Wiley, New York.
16. Tallarida, R. J., and Jacob, L. S. (1979) The Dose–Response Relationship in Pharmacology, Springer-Verlag, New York.
17. Baxter, G. T., Young, M-L., Miller, D. L., and Owicki, J. C. (1994) *Life Sci.* **55,** 573–583.
18. Gurwitz, D., Haring, R., Heldman, E., Fraser, C. M., Manor, D., and Fisher, A. (1994) *Eur. J. Pharmacol.* **267,** 21–31.