0026-895X/92/030561-08$03.00/0

MOLECULAR PHARMACOLOGY, 41:561-568

Neuronal "Nucleotide" Receptor Linked to Phospholipase C and Phospholipase D? Stimulation of PC12 Cells by ATP Analogues and UTP

RICHARD J. A. MURRIN and MICHAEL R. BOARDER
Department of Pharmacology and Therapeutics, University of Leicester, Leicester, LE1 9HN, UK
Received September 13, 1991; Accepted December 10, 1991

SUMMARY

We have investigated the characteristics of the receptor for ATP on neuronal cells and the involvement of phospholipase C and phospholipase D in the effector mechanisms, using PC12 rat phaeochromocytoma cells in culture. We show that the cells respond, with generation of total inositol phosphates, to ATP and adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) but not to 2-methylthioadenosine 5′-triphosphate (2MeSATP), β,γ-methylene ATP, or adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS). The largest response to ATPγS was mainly independent of extracellular calcium, had an EC_{50} of 7.93 ± 0.76 μM, and was competitively inhibited by the nonspecific antagonist suramin. The pyrimidine nucleotide UTP also elicited a response in these cells. Measurement of [^{3}H]inositol triphosphate showed a rapid rise to maximum (10–15 sec) in response to both ATPγS and UTP but no response to 2MeSATP. Cells prelabeled with $^{32}P_i$ and stimulated in the presence of 50 mM butanol responded to ATPγS, ATP, and UTP with enhanced formation of [^{32}P]phosphatidylbutanol as well as [^{32}P]phosphatidic acid, indicating that agonist-stimulated phosphatidic acid occurs by both phospholipase D and phospholipase C activity. The stimulation of phospholipase D was inhibited by the presence of a protein kinase C inhibitor, Ro 31-8220. The dose-response curve for the stimulation by ATPγS of phospholipase C was shifted to the right by the presence of UTP, indicating that both compounds act on the same receptors. The data provide the first evidence for the existence of a nucleotide receptor on neuronal cells (insensitive to both purines and pyrimidines) and show that this receptor is linked to both phospholipase C and phospholipase D.

Studies on receptors for ATP and ADP (P_2-purinergic receptors) have been most vigorously pursued in recent years using turkey erythrocyte preparations and vascular endothelial cells (1, 2). However, the existence of such receptors on neuronal cells is of considerable interest with respect to a possible role in neuronal regulation for ATP/ADP of either neuronal or non-neuronal origin. The two most widely used models of peripheral neurons in culture, the adrenal chromaffin cell and the rat phaeochromocytoma-derived PC12 cell line, both show evidence of cell surface receptors for ATP. Chromaffin cells are reported to respond with stimulation of inositol (poly)phosphate formation and mobilization of intracellular calcium (3, 4). With PC12 cells, ATP has been shown to elicit an inward current (5), calcium influx and $InsP_3$ accumulation (6) and [^{3}H]noradrenaline release (7).

Although apparently indicating that these actions are mediated by P_2-purinergic receptors (and not the P_1 receptors responding to adenosine and AMP), these studies do not elucidate the subtype of receptors involved. In the absence of selective antagonists, P_2-purinergic receptor classification is based on relative agonist potencies. A rank order of α,β-methylene-ATP = β,γ-methylene-ATP > ATP = 2MeSATP is characteristic of P_{2X} receptors, whereas 2MeSATP > ATP > α,β-methylene-ATP = β,γ-methylene-ATP is characteristic of P_{2Y} subtype (8). However, an increasing number of studies show responses that do not fit this classification. Of particular interest in the present context is, firstly, that there are reports of responses that are not sensitive to either 2MeSATP or β,γ-methylene ATP (9, 10) and, secondly, that these responses are, at least in some cases, also elicited by UTP (10, 11). If these non-P_{2X} and non-P_{2Y} responses to the purine ATP are mediated through the same receptors as the pyrimidine UTP responses, as has been argued for some cell types (see Ref. 12), then the term "nucleotide receptor" may be more appropriate than purinergic receptor (11, 12).

In the present report, we have investigated these emerging issues of receptor classification in a neuronal model, the PC12 cell. We have used the generation of inositol phosphates by

This work was supported by The Wolfson Foundation and The Wellcome Trust.

ABBREVIATIONS: 2MeSATP, 2-methylthioadenosine 5′-triphosphate; ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PMA, phorbol myristate acetate; $InsP_1$, inositol monophosphate of unspecified isomerism; $InsP_2$, inositol bisphosphate; $InsP_3$, inositol trisphosphate; $InsP_4$, inositol tetrakisphosphate; BSS, balanced salt solution.

phospholipase C to characterize the response to different agonists, we have investigated the participation of phospholipase D in the receptor effector mechanism, and we present data relating to the question of whether UTP and the metabolically stable ATP analogue ATPγS act at the same receptor.

Materials and Methods

Cell culture. PC12 cells, obtained from Dr. B. Spruce, Imperial College (London), were grown in Dulbecco's modified medium supplemented with 10% horse serum, 5% fetal calf serum, 27 mg/100 ml glutamine, 2500 IU/100 ml penicillin, and 2500 μg/100 ml streptomycin, plated into 24-well Nunc multiwells, and maintained in a water-saturated 95% air/5% CO_2 atmosphere at 37°. Experiments were performed on cells that had just reached confluence.

[^{3}H]Inositol phosphate formation. For study of total [^{3}H]inositol phosphates, cells were loaded for 48 hr with *myo*-[2-^{3}H]inositol at 0.074 MBq/ml, in a volume of 0.5 ml of medium composed of medium M199 with 5% fetal calf serum, 25 IU/100 ml penicillin, 2500 μg/100 ml streptomycin, and 27 mg/100 ml glutamine. Preliminary experiments showed that under these conditions the incorporation of label into cell lipids reached a plateau after 40 hr (data not shown). Experiments were undertaken with a BSS of 125 mM NaCl, 5.4 mM KCl, 16.2 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 5.5 mM glucose, 30 mM HEPES, 1 mM NaH_2PO_4, and 1.8 mM $CaCl_2$, pH 7.4, gassed with 5% CO_2/95% O_2, including, where appropriate, 10–30 mM LiCl, with a commensurate reduction in NaCl. Cells were washed in BSS; lithium was present for 10 min before and during the 30-min stimulation, in 0.5 ml, at 37°. The reaction was terminated by addition of 0.5 ml of ice-cold methanol and the cells were scraped and extracted into chloroform. The [^{3}H]inositol phosphates in the aqueous layer were batch purified on Dowex-1 (Cl^-) before counting.

For measurement of separated inositol (poly)phosphates, cells were grown in six well (3.5 cm) multiwells and loaded as described above but with 0.122 MBq/ml [^{3}H]inositol, in a volume of 1.5 ml. Cells were washed in BSS and incubated with agonists for the times shown, at 37°, in the absence of lithium. Cells were extracted into 0.5 M trichloracetic acid, the acid was removed by ether extraction, and the inositol (poly)phosphates were separated on small AG1 anion exchange columns, as described previously (9). Each inositol phosphate fraction was eluted in 12 ml; 2 ml of this were counted and the results are expressed as dpm/2 ml.

Formation of [^{32}P]phosphatidic acid and [^{32}P]phosphatidylbutanol. Cells were loaded for 24 hr with $^{32}P_i$ at 0.25 MBq/ml (0.4 ml/well), in phosphate-free BSS, as described in Ref. 13. Butanol, where appropriate, was present at 50 mM for 10 min before and during the stimulation. Phosphate was returned to the cells, to a concentration of 1 mM, 1 min before the beginning of the stimulation, which was usually for 5 min at 37° in 0.5 ml and was terminated by addition of methanol. The cells were scraped and extracted into chloroform. The organic layer was dried under nitrogen and separated on silica gel G thin layer chromatography plates developed in ethyl acetate/acetic acid/trimethylpentane (9:2:5). Radioactive spots were visualized by autoradiography, scraped, and counted. Preliminary experiments established that, in the absence of butanol, ATPγS stimulated the formation of a single radioactive spot, which chromatographed with the 1-steroyl-2-arachidonyl phosphatidic acid standard. This was the only major radioactive band running free of the radioactivity associated with the origin. Its confirmation as [^{32}P]phosphatidic acid was by deacylation, followed by high pressure liquid chromatography of the resultant glycerophosphate, as described in Ref. 13.

Agonist-stimulated [^{32}P]phosphatidic acid can originate from the sequential action of phospholipase C and diacylglycerol kinase or directly from the action of phospholipase D. In the presence of alcohols such as butanol, phospholipase C forms [^{32}P]phosphatidylbutanol, and thus this route can be distinguished from the phospholipase C route, which will be unchanged. Consistent with this, when butanol was included in the incubation a second radioactive band appeared, running in advance of phosphatidic acid, in the same position as [^{14}C]phosphatidylbutanol. Both with and without stimulation, the radioactivity in this band increased with higher butanol concentrations, reaching a plateau at 30–50 mM butanol. Phosphatidylbutanol is formed exclusively by the transphosphatidylation reaction catalyzed by phospholipase D (14–16). When incubated with 100 nM PMA, which will stimulate phospholipase D and not phospholipase C, 80–100% of the radioactivity is converted from phosphatidic acid to phosphatidylbutanol (see Results), indicating that most or all of the product of phospholipase D activity is diverted to the butanol derivative. Therefore, as used here, in the presence of 50 mM butanol the [^{32}P]phosphatidic acid formed is an index of phospholipase C activity, whereas the [^{32}P]phosphatidylbutanol represents the product of phospholipase D.

Materials. Cell culture medium, additives, and plastics were from GIBCO (Paisley, Scotland). *myo*-[2-^{3}H]Inositol was from New England Nuclear (Stevenage, Herts, UK) and [^{32}P]phosphorus from Amersham International (Buckinghamshire, UK). 2MeSATP was from Research Biochemicals (Semat Ltd., St. Albans, UK). Other chemicals and drugs were from Sigma Chemical Co. (Poole, Dorset, UK) or Fisons plc (Loughborough, UK), except for suramin (a kind gift from Dr. M. Collis, ICI Pharmaceuticals, Alderley Park, UK) and Ro 31-8220 (a kind gift from Dr. J. Lawton, Roche Products, Welwyn Garden City, UK). Thin layer chromatography plates (LK 6D) were from Whatman (Kent, UK).

Results

Characteristics of total inositol phosphate formation. When [^{3}H]inositol-loaded cells were stimulated in the presence of lithium and total inositol phosphates were separated and counted, the results obtained from a series of experiments with a variety of agonists at 30 μM each were pooled and are shown in Fig. 1. Both ATP and UTP elicited a substantial response at 30 μM; however, UDP, UMP, uridine, AMP, and adenosine gave no response, with ADP giving a significantly smaller response than ATP. These results show that the effects of ATP and UTP were not due to their breakdown to these other compounds and also that the responses are not due to action at P_1-purinergic receptors, because these would preferentially respond to adenosine and AMP. Investigations with various ATP analogues (Fig. 1B) showed that at 30 μM ATPγS gave a substantially larger response than ATP but that the P_{2X}-selective agonist, β,γ-methylene ATP and the P_{2Y}-selective agonists 2MeSATP and ADPβS failed to elicit a response. Incubation with 30 μM ATPγS (with 10 mM lithium) for increasing periods of time gave a linear relationship through 40 min, as illustrated in Fig. 2A. Incubation with 30 μM UTP for increasing periods of time under these conditions repeatedly gave a complex time course of total [^{3}H]inositol phosphate accumulations. However, when the lithium was increased to 20 or 30 mM, UTP also gave a linear accumulation of [^{3}H]inositol phosphates through 40 min (data not shown), so in subsequent experiments with UTP the high lithium concentrations were used, as indicated in the figure legends.

A series of dose-response studies with four potential agonists between 0.3 and 300 μM gave an EC_{50} for ATPγS of 7.93 ± 0.76 μM (mean ± standard error, eight experiments). ATPγS typically gave a plateau around 30 μM (see examples in Figs. 2B, 3B, and 8). Responses to UTP and ATP failed to form a plateau with increasing concentrations. In both cases, the responses began at 1 μM and increased through 30 μM but typically showed a steepening of the curve through 100 and 300 μM (data not shown), as previously reported for ATP (9) using different cell

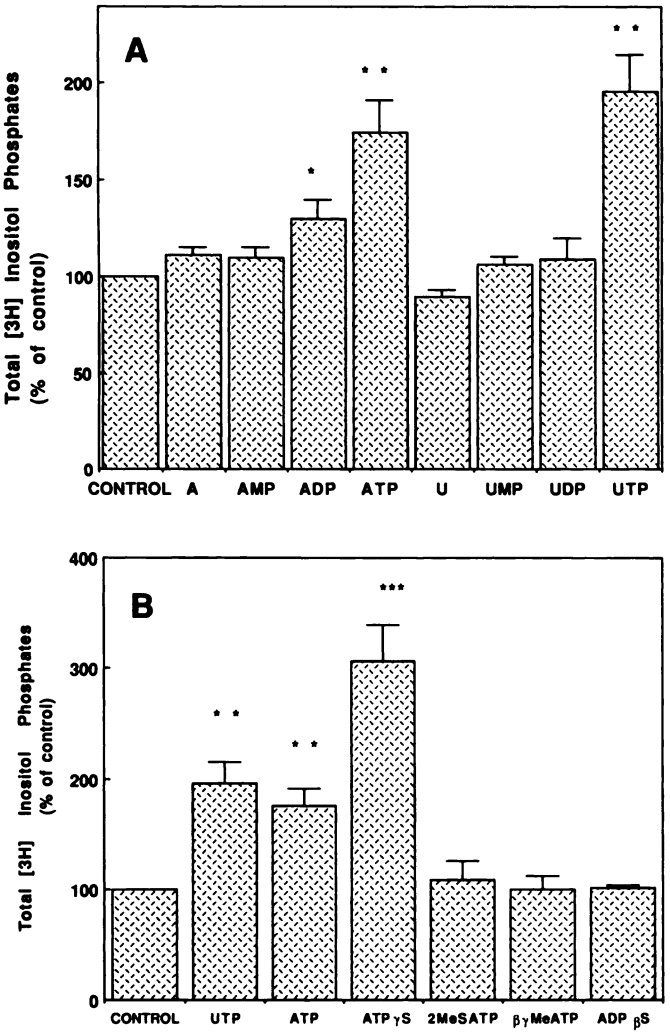

Fig. 1. Formation of total [^{3}H]inositol phosphates in PC12 cells in the presence of various agonists at 30 μM. *A*, Adenosine; *U*, uridine. Stimulations were for 30 min with 10 mM LiCl. Data are normalized to percentage of control, pooled across four separate experiments (each with triplicate determinations), and expressed as mean ± standard error. Significantly different from control: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student *t* test).

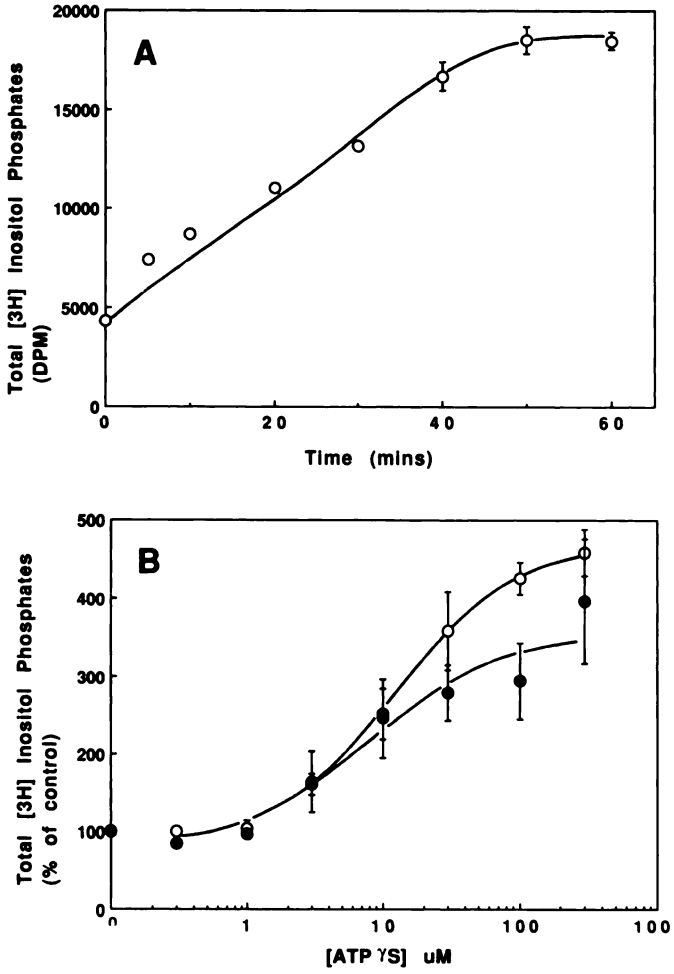

Fig. 2. Characteristics of stimulation of total [^{3}H]inositol phosphates in PC12 cells in response to ATPγS in 10 mM LiCl. A, Time course of stimulation in response to 30 μM ATPγS. Data are dpm of [^{3}H]inositol phosphates (mean ± standard error, three determinations) from one experiment representative of three. B, Dose-response curve for increasing concentrations of ATPγS with a 30-min incubation in the presence (○) and absence (●) of 1.8 mM $CaCl_2$. Data are pooled across three separate experiments (each in triplicate) and are expressed as mean ± standard error.

types. These results precluded the calculation of EC_{50} and maximal response values between the different agonists. The fourth compound for which dose-response studies were performed was 2MeSATP, which failed to give any response between 0.3 and 100 μM.

Fig. 2B also shows the dependency of the response to ATPγS on extracellular Ca^{2+}. Cells were stimulated either in the presence of 1.8 mM Ca^{2+} or in medium in which there was no added Ca^{2+}, leaving residual low micromolar concentrations. It can be seen that the nominally Ca^{2+}-free medium had little effect on the rising part of the curve but showed a tendency to reduce the response at the higher range of concentrations.

Lacking selective antagonists to the different P_2-purinergic receptors, we used the nonselective competitive antagonist suramin to confirm the nature of the ATPγS response. Fig. 3A shows that the response to 30 μM ATPγS was sensitive to suramin in the range 30 to 300 μM, with the latter bringing about a complete loss of response. Fig. 3B confirms that this is competitive inhibition, by showing dose-response curves to ATPγS in the presence and absence of 100 μM suramin. The data are consistent with a parallel shift to the right in the presence of suramin.

Separation of inositol (poly)phosphates. The nature of the inositol phosphate(s) response to ATPγS was further examined by separating the individual inositol phosphates using small ion exchange columns. The experiments involved stimulations for 0–300 sec and were undertaken for UTP and 2MeSATP, as well as ATPγS. Fig. 4 shows that, in response to ATPγS, $InsP_3$ rose very rapidly to a peak at 5–15 sec, with $InsP_2$ and $InsP_1$ reaching a maximum response at 60 and 120 sec, respectively. $InsP_4$ also showed a rapid rise in response to ATPγS, following closely behind $InsP_3$, but the subsequent level was more sustained, with considerable variation between experiments. Fig. 5 shows typical responses to UTP; these gave a much smaller increase that was more difficult to characterize. However, once again $InsP_3$ increased rapidly and before $InsP_2$. $InsP_4$ failed to show a measurable rise in response to UTP (data not shown). Of three further experiments carried out in parallel with those above using 30 μM 2 MeSATP, none showed

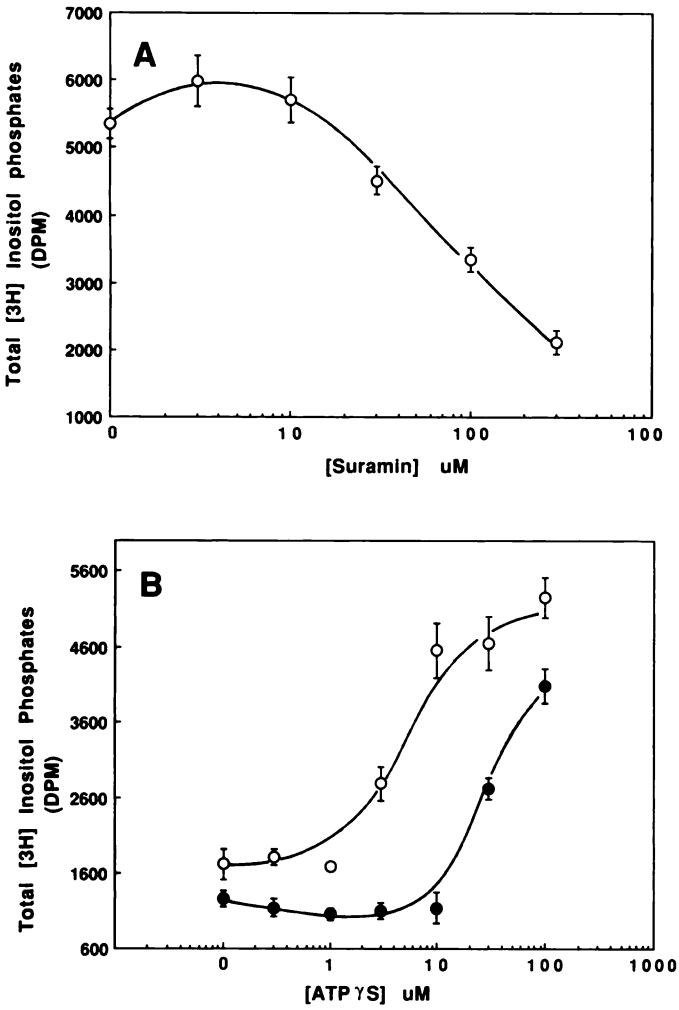

Fig. 3. Antagonism by suramin of ATPγS stimulation of [^{3}H]inositol phosphate formation. Incubations were for 30 min in the presence of 10 mM LiCl. A, Stimulation with 30 μM ATPγS with increasing concentrations of suramin. B, Stimulation with increasing concentrations of ATPγS in the presence (●) or absence (○) of 100 μM suramin. In each case, data are dpm of [^{3}H]inositol phosphate formed (mean ± standard error, three determinations) from one experiment representative of three.

any increase in the levels of the inositol (poly)phosphates (data not shown).

Stimulation of Phospholipase D. The possible involvement of phospholipase D was investigated by labeling cells with $^{32}P_i$ and then stimulating them in the presence of 50 mM butanol. Under these conditions [^{32}P]phosphatidic acid is still formed by sequential phospholipase C/diacylglycerol kinase action, but the product of phospholipase D is [^{32}P]phosphatidylbutanol. These two responses are shown in Table 1, in response to various agonists and to the protein kinase C-stimulating phorbol ester PMA, which is reported to stimulate phospholipase D in a wide variety of cells. Indeed, PMA produced a large stimulation of phospholipase D (Table 1) but no effect on phosphatidic acid. Of the agonists tested, ATPγS produced the largest stimulation of [^{32}P]phosphatidylbutanol accumulation, with ATP and UTP also producing substantial responses. However, neither β,γ-methylene ATP nor 2MeSATP produced any stimulation of phospholipase D. These results are also reflected in the [^{32}P]phosphatidic acid accumulation, with ATPγS, ATP, and UTP giving a response but β,γ-methylene ATP and 2MeSATP being ineffective.

Fig. 6 shows the effect of stimulation with ATPγS in the presence of increasing concentrations of the relatively specific protein kinase C inhibitor Ro 31-8220 (17). ATPγS produced the anticipated enhancement of [^{32}P]phosphatidylbutanol, which was effectively inhibited, characteristically to just below control levels, in the presence of 10 μM Ro 31-8220. However, the agonist-stimulated [^{32}P]phosphatidic acid formation was unaffected by this agent (Fig. 6).

Relationship between UTP and ATP. To investigate whether UTP was acting on the same receptor population as ATP and ATPγS, two sets of experiments were undertaken, using accumulation of total inositol phosphates in the presence of lithium as the response. Firstly, simple additivity experiments were performed, as shown in Table 2. The effect of adding UTP as well as ATP or ATPγS was to bring about no further increase in response beyond that caused by the purine nucleotides alone. This is consistent with the notion that the population of receptors is the same. To pursue this further, we obtained dose-response curves for ATPγS in the presence or absence of 30 μM UTP. We found that, in each of three experiments undertaken, the dose-response curve for ATPγS was shifted substantially to the right. This was clearly seen when the data from all three experiments were pooled, as shown in Fig. 7. In the absence of UTP the EC_{50} values for these three experiments were 7.50 ± 1.26 μM, and in the presence of UTP the values were 40.1 ± 20.9 μM (mean ± standard error, three experiments). The maximum response to ATPγS was not increased in the presence of UTP (as in Table 2), so the range of the ATPγS curve was reduced because it started from a higher level; this is also apparent in Fig. 7.

Discussion

Is the response to purines at P_{2X} or P_{2Y} receptors? The division of P_2-purinergic receptors into P_{2X} and P_{2Y} subtypes on the basis of agonist potency profiles (8) has been retained as the basis of P_2-purinergic classification, despite the proliferation of other putative receptor subtypes. These have included those proposed for specific cell types, notably the P_{2T} receptor for platelets and the P_{2Z} receptor for mast cells (18). However, there have also been a number of reports in diverse cell types that do not fit the pattern of agonist responses expected of P_{2X} or P_{2Y} (9, 10). It is apparent that the responses to purinergic agonists in the present report show that PC12 cells should be included among these. This appears to be the first indication that such non-P_{2X} non-P_{2Y} responses are found in neuronal cells. The salient observation giving rise to this conclusion is that the responses are sensitive to neither the P_{2X} agonist β,γ-methylene ATP nor the P_{2Y} agonists 2MeSATP and ADPβS. A possible role of breakdown should be considered before this conclusion is accepted. This largest response, of the agonists tested, is to ATPγS, a relatively stable analogue. Chromatographic analyses carried out in our laboratory[1] show that substantial breakdown of ATP and 2MeSATP (as well as UTP) does occur during the course of the 30-min incubations we used for the total inositol phosphate studies. However, the following points clearly indicate that breakdown cannot account for the failure of the cells to respond to P_{2X}- and P_{2Y}-specific analogues: 1) β,γ-methylene ATP is stable under these conditions; 2) of the two P_{2Y} agonists that failed to elicit a

[1] Unpublished observations.

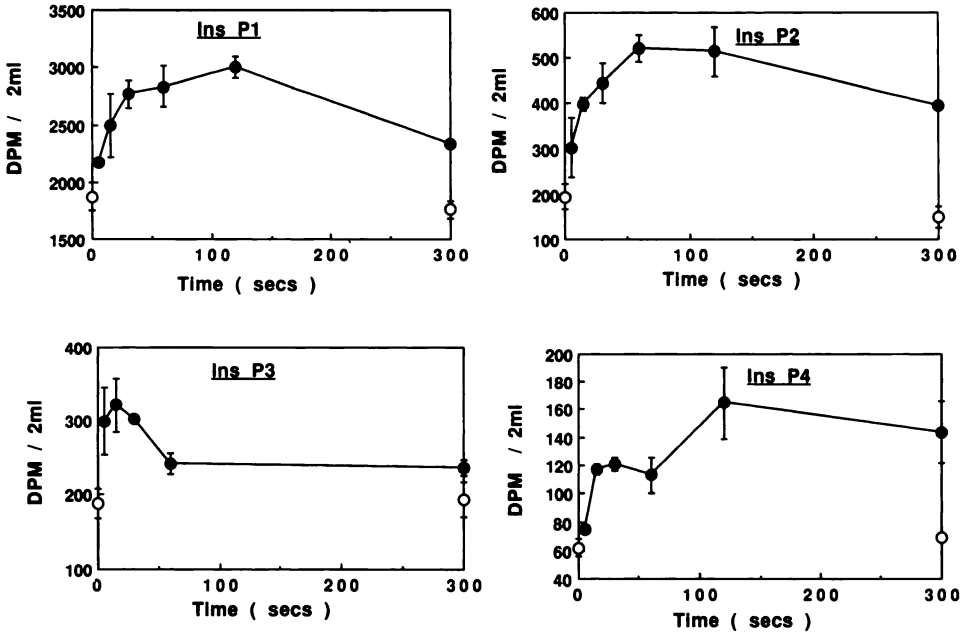

Fig. 4. Formation of separated [^{3}H]inositol (poly)phosphates in response to stimulation with 30 μM ATPγS. Data are mean ± standard error (three determinations) from one experiment (in each case) representative of three. ○, Controls; ●, stimulated.

response, ADPβS is relatively resistant to breakdown; 3) the nondiscriminative agonist ATP, which is as susceptible to breakdown as is the P_{2Y}-selective 2MeSATP, does elicit a response; and 4) no response to 2MeSATP was seen even when the experiment involved short incubation times. The latter point is relevant with respect to the 5-min incubation during the phospholipase D experiments but is even more important in the measurement of separated $InsP_3$, in which a few seconds of incubation produce a maximal response with ATPγS (and UTP) but there is no response to 2MeSATP. Although it seems likely that breakdown plays a role in determining the relative size of responses in the long incubation for total inositol phosphates (perhaps accounting for some of the differences between ATP and ATPγS), the following conclusion is not compromised: the response of PC12 cells to purinergic agonists cannot be accounted for by action at either P_{2X} or P_{2Y} receptors.

The importance of the suramin experiments reported here is in the confirmation that the ATPγS response does depend upon action at cell surface receptors with predictable pharmacology. Of course, the use of selective antagonists would be more informative, but in their absence the suramin studies show that the ATPγS response can be inhibited in a competitive manner, as predicted from other studies with this antagonist (19, 20).

Is there a neuronal "nucleotide" receptor? The introduction of the pyrimidine UTP into the studies involving so called purinergic receptors has raised a number of questions. Investigations into responses to UTP now have a substantial

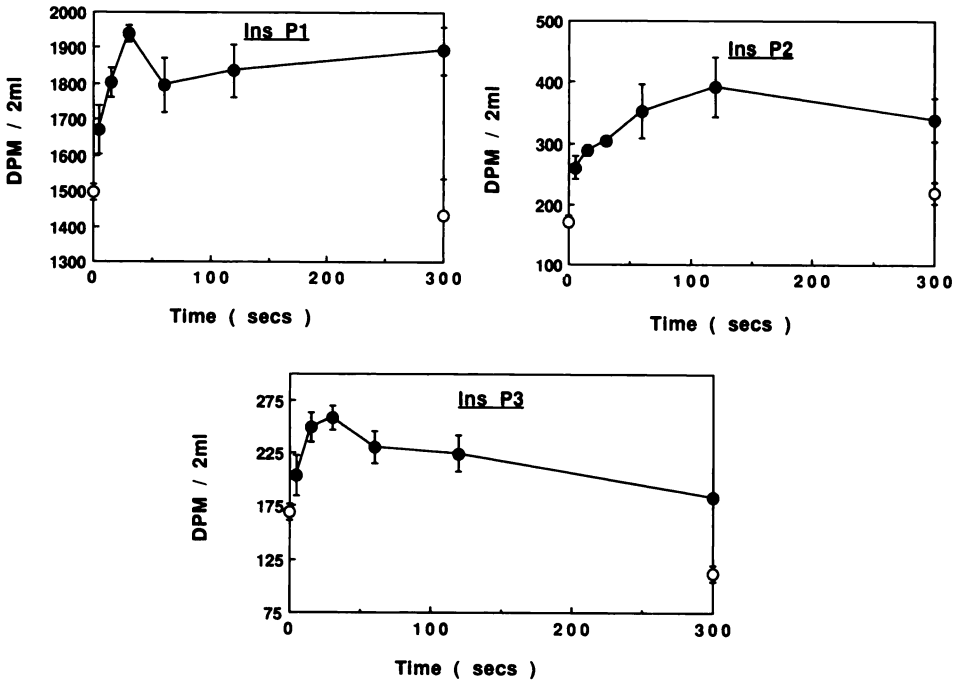

Fig. 5. Formation of separated [^{3}H]inositol (poly)phosphates in response to stimulation with 30 μM UTP. Data are mean ± standard error (three determinations) from one experiment representative of three. ○, Controls; ●, stimulated.

TABLE 1

Stimulation of phosphatidylbutanol and phosphatidic acid formation by various agonists

Cells were stimulated for 5 min with the nucleotides indicated at 30 μM or PMA at 100 nM. Data are the percentage of unstimulated control expressed as mean ± standard error of three separate experiments (each in triplicate). Typical cpm were, in phosphatidylbutanol, 2,218 ± 68 (control) and 10,312 ± 752 (30 μM ATPγS) and, in phosphatidic acid, 2,908 ± 400 (control) and 6,202 ± 1,405 (30 μM ATPγS).

	Formation	
	[^{32}P]Phosphatidylbutanol	[^{32}P]Phosphatidic acid
	% of control	
PMA	504 ± 41[a]	85 ± 11
ATPγS	439 ± 46[a]	261 ± 34[b]
ATP	327 ± 54[a]	174 ± 32[c]
UTP	335 ± 33[a]	144 ± 9[b]
β,γ-Methylene ATP	102 ± 7	94 ± 32
2MeSATP	115 ± 11	87 ± 17

[a–c] Significance of difference from controls: [a] $p < 0.001$, [b] $p < 0.01$, [c] $p < 0.05$ (Student's *t* test).

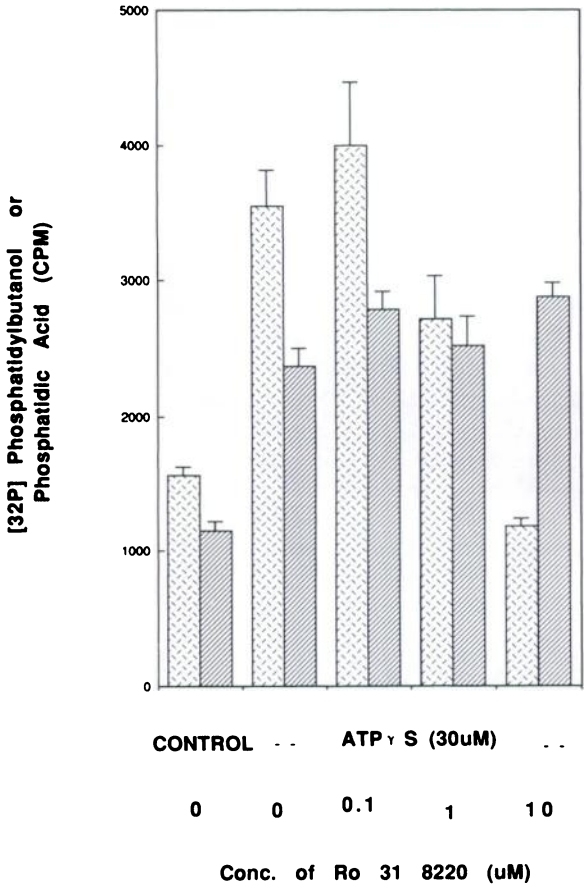

Fig. 6. Stimulation of [^{32}P]phosphatidic acid (▨) and [^{32}P]phosphatidylbutanol (▩) by ATPγS (30 μM) for 5 min in the presence of the protein kinase C inhibitor Ro 31-8220. The inhibitor was present 10 min before and during the 5-min incubation period. Data are mean ± standard error (three determinations) from one experiment representative of three.

TABLE 2

Formation of total [^{3}H]inositol phosphates stimulated by ATP and ATPγS in the presence or absence of UTP

All agonists were at 30 μM for a 15-min stimulation in the presence of 30 mM LiCl. Data are mean ± standard error (three determinations) from one representative experiment of three.

	[^{3}H]Inositol phosphate formation		
	Control	ATP	ATPγS
	dpm		
Control	3,478 ± 479	5,637 ± 245[a]	10,295 ± 468[b]
UTP	6,769 ± 275[c]	5,500 ± 194	10,345 ± 647[b]

[a–c] Significant difference from unstimulated controls: [a] $p < 0.05$, [b] $p < 0.001$, [c] $p < 0.01$.

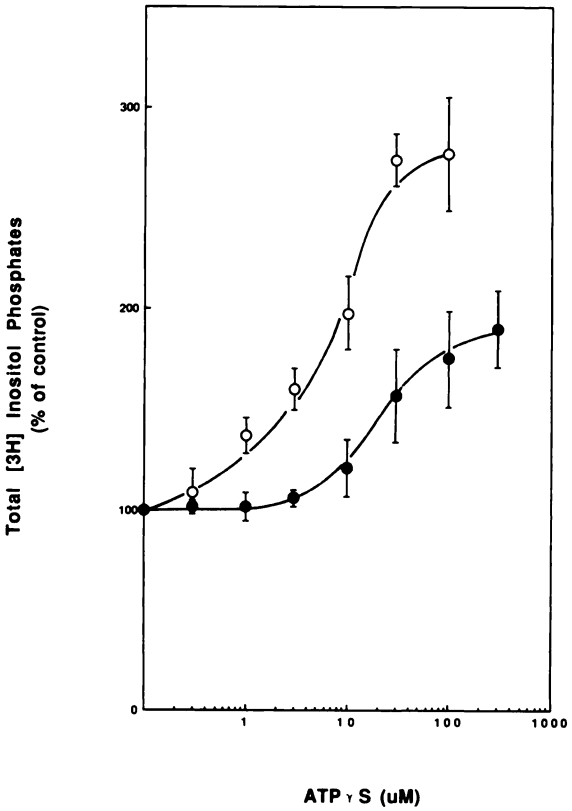

Fig. 7. Dose-response curve for ATPγS-stimulated formation of total [^{3}H]inositol phosphates in PC12 cells in the presence (●) or absence (○) of 30 μM UTP. Incubations were for 15 min in the presence of 30 mM LiCl. Data are expressed as mean ± standard error of three experiments (each in triplicate).

background, and one of the questions such responses raised from the start was whether there was a specific "pyrimidinergic" receptor or whether UTP acts on receptors hitherto described as purinergic. There is some apparent diversity, between tissue and cell types, in the answer to this question (21).

The definitive pharmacological answer to this question with respect to PC12 and other cells in culture will probably await the application of a suitable radioligand binding assay along with more selective analogues. However, the following points arise from the data presented here: 1) the response measured here showed no dissociation between purinergic (ATP and ATPγS) and pyrimidinergic (UTP) responses; 2) there was no apparent additivity between the responses elicited by UTP and the purines; and 3) the dose-response curve for ATPγS was shifted to the right by the presence of UTP.

The interpretation of the additivity experiment is confused by the failure to reach saturation in dose-response curves for UTP and ATP. Despite this, these experiments are still possible because ATPγS did show a plateau in its dose-response curve. The observation that UTP does not increase the response above the maximally effective concentration of ATPγS suggests that they are acting upon the same overlapping receptor populations. Again, the failure to generate saturating response curves for UTP and ATP restricted the design of dose-response experi-

ments to the use of ATPγS in the presence and absence of UTP. This experimental design is based upon the prediction that, if the two agonists were acting on different receptors, then the EC_{50} for ATPγS would be unaffected by the presence of UTP. However, if they were acting on the same receptor population, then UTP would cause an increase in the EC_{50} for ATPγS. The observation that UTP caused a rightward shift in the concentration-response curve for ATPγS clearly indicates that these two are acting on the same receptors. Comparison of the size of the movement of the ATPγS curve with the theoretical prediction is not possible in the absence of a saturating dose-response curve for UTP or knowledge of receptor occupation by the two agonists. In conclusion, however, the data provide a strong indication that ATPγS and UTP are acting on the same receptors and, therefore, there may be a neuronal "nucleotide" receptor of the type tentatively proposed for other cell types (11, 12).

Activation of phospholipase D. Earlier studies have shown that bradykinin may stimulate phospholipase D in PC12 cells (22, 23). The studies reported here involved the labeling of phospholipids, by the incubation of cells with $^{32}P_i$, and subsequent analysis of the formation of phosphatidic acid. Using this procedure, agonist-stimulated [^{32}P]phosphatidic acid may originate from one of two sources, either by sequential action of phospholipase C and diacylglycerol kinase or directly by action of phospholipase D. Using the procedure in the presence of 50 mM butanol distinguishes between these, in that the product of the phospholipase D activity is [^{32}P]phosphatidylbutanol. Here we show that activation of phospholipase D follows the same agonist profile as activation of phospholipase C. Principally, ATP, ATPγS, and UTP elicit responses, whereas β,γ-methylene ATP and 2MeSATP are ineffective. This shows that phospholipase D activation has the same pharmacological characteristics as phospholipase C, namely non-P_{2X} and non-P_{2Y} but UTP sensitive, and this raises the question of whether phospholipase D activation is downstream of phospholipase C. Phospholipase D is stimulated by PMA, so one possible mechanism for agonist activation of phospholipase D is via protein kinase C activation as a consequence of agonist-stimulated formation of diacylglycerol by phospholipase C. Here, use of a relatively specific protein kinase C inhibitor (17) indicates that the agonist stimulation of phospholipase D is dependent on protein kinase C. This suggests that the primary receptor effector mechanism is phospholipase C and that phospholipase D activation is part of the cascade following activation of this enzyme. The final lipid second messenger in these two pathways may be either phosphatidic acid or diacylglycerol or both, because there is interconversion between diacylglycerol and phosphatidic acid by diacylglycerol kinase and phosphatidate phosphohydrolase.

This interpretation of the butanol procedure assumes that all the phospholipase D product is converted to phosphatidylbutanol. The preliminary experiment (data not shown) indicating that at 50 mM we have reached saturating levels of butanol may be interpreted to support this view, but the direct demonstration that this is so lies in the observation that PMA stimulation in the presence of 50 mM butanol leads to stimulated levels of phosphatidylbutanol but not of phosphatidic acid. The observation that there is agonist-stimulated phosphatidic acid as well as phosphatidylbutanol indicates, therefore, that the stimulation of these cells by purines/pyrimidines will generate phosphatidic acid by two routes, sequential phospholipase C/diacylglycerol kinase and phospholipase D.

General conclusions. The PC12 neuronal cells have receptors for ATP that conform to neither P_{2X} nor P_{2Y} characterization. Evidence presented supports the view that these receptors respond to both purines and pyrimidines and, therefore, provides the first evidence for neuronal nucleotide receptors. These receptors are linked directly to phospholipase C. Phospholipase D is also activated, probably downstream of phospholipase C and protein kinase C. Stimulation leads to formation of phosphatidic acid by both phospholipase C and phospholipase D pathways. The significance may lie in phosphatidic acid levels themselves regulating cell function (24–26) or in regulation of diacylglycerol levels. Phospholipase D has been described in synaptic membranes and synaptosomes (e.g., see Refs. 27, 28). The present results indicate that extracellular ATP may regulate neuronal processes through a nucleotide receptor and that this may be effected by both phospholipase C and phospholipase D.

Acknowledgments

We thank Mrs. Lyn McCarthy for manuscript preparation.

References

1. Cooper, C. L., A. J. Morris, and T. K. Harden. Guanine nucleotide-sensitive interaction of radiolabeled agonist with phospholipase C-linked P_2-purinergic receptors. *J. Biol. Chem.* **264**:6202–6206 (1989).
2. Martin, T. W., and K. Michaelis. P_2-purinergic agonists stimulate phosphodiesteratic cleavage of phosphatidylcholine in endothelial cells: evidence for activation of phospholipase D. *J. Biol. Chem.* **264**:8847–8856 (1989).
3. Sasakawa, N., T. Nakaki, S. Yamamoto, and R. Kato. Stimulation by ATP of inositol triphosphate accumulation and calcium mobilisation in cultured adrenal chromaffin cells. *J Neurochem.* **52**:441–447 (1988).
4. Kim, K.-T., and E. W. Westhead. Cellular responses to Ca^{2+} from extracellular and intracellular sources are different as shown by simultaneous measurements of cytosolic Ca^{2+} and secretion from bovine chromaffin cells. *Proc. Natl. Acad. Sci. USA* **86**:9881–9885 (1989).
5. Nakazawa, K., K. Fujimori, A. Takanaka, and K. Inoue. Reversible and selective antagonism by suramin of ATP-activated inward current in PC12 phaeochromocytoma cells. *Br. J. Pharmacol.* **101**:224–226 (1990).
6. Fasolato, C., P. Pizzo, and T. Pozzan. Receptor mediated calcium influx in PC12 cells. *J. Biol. Chem.* **265**:20351–20355 (1990).
7. Inoue, K., K. Nakazawa, M. Ohara-Imaizumi, T. Obama, K. Fujimori, and A. Takanaka. Selective and competitive antagonism by suramin of ATP-stimulated catecholamine secretion from PC12 phaeochromocytoma cells. *Br. J. Pharmacol.* **102**:581–584 (1990).
8. Burnstock, G., and C. Kennedy. Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.* **16**:433–440 (1985).
9. Allsup, D. J., and M. R. Boarder. Comparison of P_2 purinergic receptors of aortic endothelial cells with those of adrenal medulla: evidence for heterogeneity of receptor subtype and of inositol phosphate response. *Mol. Pharmacol.* **38**:84–91 (1990).
10. Demolle, D., C. Lagneau, and J. M. Boeynaems. Stimulation of prostacyclin release from aortic smooth muscle cells by purine and pyrimidine nucleotides. *Eur. J. Pharmacol.* **155**:339–343 (1988).
11. Davidson, J. S., I. K. Wakefield, U. Sohnius, P. A. van der Merwe, and R. P. Millar. A novel extracellular nucleotide receptor coupled to phosphoinositide C in pituitary cells. *Endocrinology* **126**:80–87 (1990).
12. O'Connor, S. E., I. A. Dainty, and P. Leff. Classification of P_2 purinoceptors: trends emerging from recent agonist-based studies. *Trends Pharmacol. Sci.* **12**:137–141 (1991).
13. Owen, P. J., and M. R. Boarder. Influence of bradykinin on diacylglycerol and phosphatidic acid formation in cultured adrenal chromaffin cells. *J. Neurochem.* **57**:760–768 (1991).
14. Kobayashi, M., and J. N. Kanfer. Phosphatidylethanol formation via transphosphatidylation by rat brain synaptosomal phospholipase D. *J. Neurochem.* **48**:1597–1603 (1985).
15. Pai, K. J., M. I. Siegel, R. W. Egan, and M. M. Billah. Phospholipase D catalyzes phospholipid metabolism in chemotactic peptide-stimulated HL-60 granulocytes. *J. Biol. Chem.* **263**:12472–12477 (1988).
16. Bonser, R. W., N. T. Thompson, R. W. Randall, and L. G. Garland. Phospholipase D activation is functionally linked to superoxide generation in the human neutrophil. *Biochem. J.* **264**:617–620 (1989).
17. Davis, P. D., C. H. Hill, E. Keech, G. Lawton, J. S. Nixon, A. D. Sedgwick, J. Wadsworth, D. Westmacott, and S. E. Wilkinson. Potent selective inhibitors for protein kinase C. *FEBS Lett.* **259**:61–63 (1989).

18. Gordon, J. L. Extracellular ATP: effects, source and fate. *Biochem. J.* **233**:309–319 (1988).
19. Dunn, P. M., and A. G. Blakeley. Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.* **93**:827–828 (1987).
20. Hoyle, C. H. V., G. E. Knight, and G. Burnstock. Suramin antagonises responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea pig urinary bladder and taenia coli. *Br. J. Pharmacol.* **99**:617–621 (1990).
21. Seifert, R., and G. Schultz. Involvement of pyrimidinoceptors in the regulation of cell functions by uridine and uracil nucleotides. *Trends Pharmacol. Sci.* **10**:365–369 (1989).
22. Horwitz, J. Bradykinin activates a phospholipase D that hydrolyses phosphatidylcholine in PC12 cells. *J. Neurochem.* **56**:509–517 (1991).
23. Purkiss, J. R., R. J. A. Murrin, P. J. Owen, and M. R. Boarder. Lack of phospholipase D activity in chromaffin cells: bradykinin stimulated phosphatidic acid formation involves phospholipase C in chromaffin cells but phospholipase D in PC12 Cells. *J. Neurochem.* **57**:1084–1087 (1991).
24. Barrit, G. J., K. A. Dalton, and J. A. Whiting. Evidence that phosphatidic acid stimulates the uptake of calcium by liver cells but not calcium release from mitochondria. *FEBS Lett.* **125**:137–140 (1981).
25. Moolaar, W. H., W. Kruijier, B. C. Tilly, I. Verlaan, A. J. Birman, and S. W. deLatt. Growth factor like action of phosphatidic acid. *Nature (Lond.)* **323**:171–173 (1986).
26. Murayama, T., and M. Ui. Phosphatidic acid may stimulate membrane receptors mediating adenylate cyclase inhibition and phospholipid breakdown in 3T3 fibroblasts. *J. Biol. Chem.* **262**:5522–5529 (1987).
27. Chalifa, V., H. Mohn, and M. Liscovitch. A neutral phospholipase D activity from rat brain synaptic plasma membranes: identification and characterization. *J. Biol. Chem.* **265**:17512–17519 (1990).
28. Qian, Z., and L. R. Drewes. Muscarinic acetylcholine receptor regulates phosphatidylcholine phospholipase D in canine brain. *J. Biol. Chem.* **264**:21720–21724 (1989).

Send reprint requests to: Dr. M. R. Boarder, Department of Pharmacology and Therapeutics, University of Leicester, P.O. Box 138, Medical Sciences Building, University Road, Leicester, LE1 9HN, UK.