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KINETIC STUDIES ON DOSE–TRIPHOSPHOINOSITIDE RESPONSES AND DOSE–CONTRACTION RESPONSES IN RABBIT IRIS*

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Abstract—Correlative studies on the dose–triphosphoinositide (TPI) breakdown and –phosphatidic acid (PA) labeling and dose–tension relationship to acetycholine (ACh), carbachol and acetyl-β-methacholine were investigated in the rabbit iris smooth muscle. Concentrations of these agonists between 1×10^{-8} M and 1×10^{-4} M caused a concentration-dependent TPI breakdown and PA labeling, and concentrations between 1×10^{-7} M and 1×10^{-3} M caused a concentration-dependent contraction of the iris. The ED_{50} values for the various cholinergic muscarinic agonists were determined from the dose–response curves. Good correlation between the ED_{50} values determined by the biochemical and pharmacological methods, being of the order of $1–8 \times 10^{-6}$ M, were observed. The apparent dissociation constants of ACh and atropine were estimated by measuring the effects of these agents on TPI metabolism and iris muscle contraction. Atropine, at concentrations between 1×10^{-10} M and 1×10^{-9} M, produced a parallel shift to the right of the ACh dose–response curve in both the biochemical and pharmacological methods. Both biochemical and pharmacological responses were inhibited in a competitive manner by atropine. For the TPI, PA and contraction responses, the K_B values (determined with ACh as agonist) were 1.7×10^{-10}, 2×10^{-9} M and 1.14×10^{-10} M, respectively, and the corresponding pA_2 values were 10.30, 10.35, and 9.90 respectively. It was concluded that the findings of similar K_B values for the atropine–muscarinic receptor, along with similar pA_2 values from the Schild plots, with the TPI, PA and contraction responses in the rabbit iris could suggest a close relationship between the biochemical and pharmacological responses.

In previous communications from this laboratory [1, 2] we reported that acetylcholine (ACh) and norepinephrine increased significantly the breakdown of triphosphoinositide ("TPI effect") in ^{32}P-labeled rabbit iris smooth muscle and that this TPI effect is mediated through cholinergic muscarinic and α-adrenergic receptors respectively. Furthermore, we reported that the TPI effect in response to ACh [3] and norepinephrine [4] is dependent upon the presence of Ca^{2+} in the incubation medium, and the enzyme that might be involved in this phenomenon appears to be TPI-phosphodiesterase [5]. More recently, it was shown that TPI breakdown and phosphatidic acid (PA) labeling are associated with sympathetic denervation supersensitivity in a dose-dependent manner [6]. While these findings suggest that TPI breakdown could be associated with muscle response, the precise relationship between the biochemical and pharmacological responses is still unclear.

If TPI breakdown does lie on the pathway linking the activated receptor to muscle contraction, and the binding of the agonist to the receptor is the rate-limiting step as it appears to be for other physiological responses in smooth muscle, then the kinetic constants derived from the dose–TPI responses should be in agreement with those derived from dose–contraction responses. In the present study, we have estimated the apparent dissociation constants of cholinergic agonists and atropine by measuring the effects of these agents on TPI metabolism and muscle contraction in the rabbit iris.

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MATERIALS AND METHODS

Chemicals

Acetylcholine hydrochloride, carbamycholine hydrochloride (carbachol), acetyl-β-methacholine, atropine sulfate, eserine sulfate and 2-deoxyglucose were obtained from the Sigma Chemical Co., St Louis, MO. [^{32}P]Orthophosphate, carrier free, was obtained from New England Nuclear, Boston MA. All other chemicals were reagent grade.

Determination of dose–TPI and dose–PA responses

The dose–TPI and dose–PA responses were essentially measured as described previously [1, 2, 6] and can be briefly summarized as follows.

Preparation of rabbit irises. Albino rabbits of either sex, weighing approximately 1 kg, were stunned by a blow to the head and exsanguinated. The eyes were immediately enucleated and placed in a cold modified Bradford–Tris buffer, pH 7.4 [1].

Incubation of irises. To label the phospholipids radioactively, the paired irises were transferred to 2 ml of the iso-osmotic medium that contained 25 μCi ^{32}per ml and incubated for 20 min at 37°. At the end of the incubation, the irises were washed three times with excess nonradioactive medium that contained 10 mM 2-deoxyglucose. This concentration of 2-deoxyglucose in the washing and subsequent incubation was shown in our previous work to decrease effectively the endogenous ATP level in the iris muscle and to prevent further labeling of phospholipids [1]. The ^{32}P-labeled irises were then incubated separately (one of each pair was

used as control and the other as experimental) for an additional 15 min in 1 ml of the nonradioactive medium that contained 2-deoxyglucose in the absence and presence of the cholinergic drug. In experiments containing ACh, 0.05 mM eserine (final concentration) was added along with the agonist. When the effects of atropine were studied, the blocker was added to the incubation medium 5 min before the addition of the cholinergic agonist.

Phospholipid analysis. Extraction, isolation and determination of the radioactive contents of the iris phospholipids were as described previously [1, 2]. Briefly, at the end of a 15-min incubation 1 ml of 10% (w/v) trichloroacetic acid was added to each tube. The irises were washed twice with distilled water, blotted dry, and homogenized first in chloroform–methanol–concentrated HCl (300:300:1.5), and then in chloroform–methanol–concentrated HCl (400:200:1.5). The lipid extracts were pooled and evaporated under N_2. The lipid residue was redissolved in chloroform and washed once with 0.5 ml of 0.1 N HCl. The aqueous layer was removed, and the chloroform layer was dried under a N_2 stream and redissolved in 0.05 ml chloroform in preparation for thin-layer chromatography. The individual phospholipids were isolated by means of two-dimensional thin-layer chromatography [1]. The solvent systems used were: first dimension, chloroform–methanol–NH_4OH (65:25:4); second dimension, *n*-butanol–acetic acid–water (6:1:1). The lipids were visualized using iodine vapor. For counting, the individual phospholipids of interest were scraped from the plates and placed into glass scintillation vials that contained 8 ml of the following mixture: 0.4% 2,5-diphenyloxazole (PPO) and 0.015% 1, 4-bis-(5-phenyloxazol-2-yl) benzene (POPOP) in a toluene base. All radioactive counting was done using a Beckman model LS-230 liquid scintillation counter.

Construction of dose–TPI and dose–PA responses. Dose–TPI and dose–PA response curves to ACh (or other muscarinic agonists) or ACh plus atropine were constructed as follows. The stimulatory effect of ACh in the absence and presence of atropine on TPI breakdown was expressed as the decrease in ^{32}P-radioactivity from TPI upon addition of various concentrations of the chlolinergic agents. Maximum TPI response was taken at the dose beyond which further increases in ACh produced no further increase in loss of radioactivity from TPI. On the left ordinate, response was plotted as actual percentage loss in ^{32}P-radioactivity from TPI in response to the cholinergic agents, and on the right ordinate, response was plotted as percentage of the maximum TPI response. For the PA response, the stimulatory effect of the cholinergic drugs was measured as the increase in ^{32}P-radioactivity. Maximum PA response was taken at the dose of ACh beyond which higher concentrations of the neurotransmitter produced no further increases in PA labeling. On the left ordinate, response was plotted as actual percentage increase in ^{32}P-labeling of PA in response to various concentrations of the drug; on the right ordinate, response was plotted as percentage of the maximum PA response. The maximal TPI and PA responses were taken as 100 per cent response.

Determination of dose–tension responses

The dose–tension responses were essentially measured as described previously for vascular smooth muscle [7] and can be briefly summarized as follows.

Iris muscle preparation. The rabbits were stunned by a blow to the head, exsanguinated, and the eyes were enucleated. The iris muscle was isolated from each eye and mounted in an organ chamber containing normal Ringer's solution (pH 7.2) which was continuously oxygenated (97%O_2–3% CO_2) and maintained at a constant temperature of 37°.

Incubation of irises. After mounting in the organ chamber, ligatures were placed on both ends of the muscle; one end was attached to a glass muscle holder and the other to a Grass FTO3 force–displacement transducer. The muscle preparations were equilibrated for 90 min under a resting tension of 200 mg before they were exposed to the cholinergic agents. During equilibration, the tissues were washed with fresh Ringer's solution every 20 min to prevent accumulation of metabolic end products. Isometric contractions were recorded on a Beckman type R411 Dynograph.

Determination of the contraction response. At the end of the initial equilibration period in normal Ringer's solution, dose–response relationships were determined for the cholinergic agonists.

Construction of dose–contraction response curves. The stimulatory effects of various concentrations of ACh, in the absence and presence of atropine, acetyl-β-methacholine and carbachol, on the contraction response of the iris were recorded as the increase in tension observed after addition of the agonist. Maximum response was defined as the maximum tension recorded with ACh (plotted as 100 per cent). All responses were plotted as percentages of this maximum response against the log molar concentration of agonist. These studies were run in the absence of eserine.

Calculations and determination of the kinetic constants from the biochemical and pharmacological data

In these experiments, the data were plotted as percent of the maximum biochemical (as well as percent of control) and pharmacological responses to the cholinergic agonists. From these curves, the concentration of the agonist resulting in half-maximum response (ED_{50}) was calculated. The dissociation constant for the receptor–antagonist complex (K_B) was calculated according to the equation $[A']/[A] - 1 = [B]/K_B$, where $[A']/[A]$ (= dose ratio) is the ratio of concentrations of agonist giving an equal response in the presence and in the absence, respectively, of a given concentration $[B]$ of the antagonist [8]. This requires that the values for dose ratio − 1 for the agonist against the concentration of the antagonist, plotted in their logarithmic forms (Schild plot), fit a straight line with a slope near unity [9]. In this way, also, the corresponding pA_2 value ($= -\log K_B$) was obtained [9]. Calculations for regression analysis and standard error of the mean were carried out on a Monroe 1930 calculator.

RESULTS

Dose–response curves (TPI breakdown, PA labeling and contraction responses) to various muscarinic agonists

To throw more light on the relationship between the biochemical and pharmacological responses and, thus,

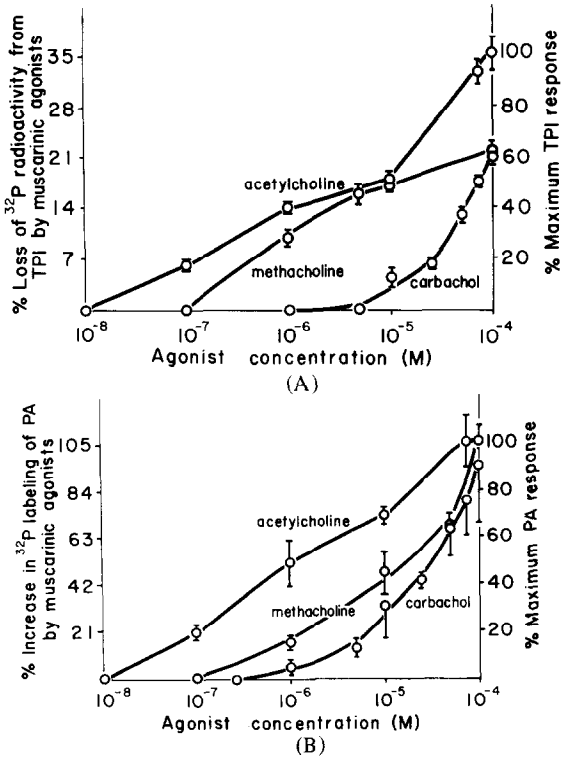


Fig. 1. Panel A: Dose–response curves (TPI breakdown) to ACh, acetyl-β-methacholine and carbachol in rabbit iris muscle.
In these experiments the irises were prelabeled in pairs with $^{32}P_i$ (25 μCi/ml in a final volume of 2 ml) for 20 min at 37°. At the end of this incubation, the irises were washed three times with non-radioactive incubation medium that contained 10 mM 2-deoxyglucose. The irises were then incubated in the non-radioactive medium plus 2-deoxyglucose in the absence and presence of various concentrations of the agonists for 10 min at 37°. The lipids were extracted, then separated by means of two-dimensional thin-layer chromatography, and their radioactive contents were determined. The TPI response to the agonist was expressed as either percent of the maximal TPI decrease in ^{32}P labeling or as percent decrease in labeling from control. Each point represents the mean of 3–5 separate determinations ± S.E.M. Panel B: Dose–response curves (PA labeling) to ACh, acetyl-β-methacholine and carbachol in rabbit iris muscle.
Conditions of incubation are described in the legend for panel A. The PA response to the agonist was expressed as either percent of the maximal PA increase in ^{32}P labeling or as percent increase in labeling over control. Each point represents the mean of 3–5 individual determinations ± S.E.M.

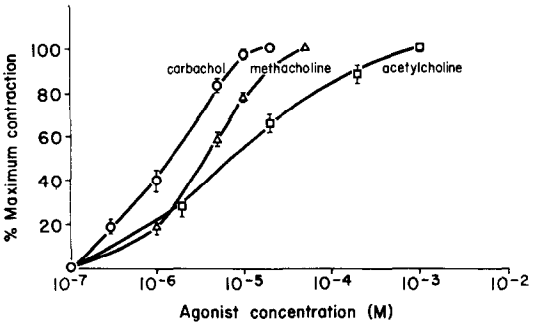


Fig. 2. Dose–response curves (contraction) to ACh, acetyl-β-methacholine and carbachol in rabbit iris muscle.
Experimental conditions were described under Materials and Methods. In short, isometric contractions to cumulative increases in the concentration of each agonist were recorded. After each recorded contraction, the muscle was allowed to re-equilibrate before the next concentration of agonist was added. Response was plotted as a percentage of the maximum recorded contraction. Each point represents the mean + S.E.M. of eight individual determinations.

to add further support to the suggestion that polyphosphoinositide turnover has an important role in the response to the muscarinic receptor, the effects of various concentrations of ACh, carbachol and acetyl-β-methacholine on the stimulation of TPI breakdown, PA labeling and muscle contraction were investigated. The dose–TPI and dose–PA responses for concentrations of the agonists between 1×10^{-8} M and 1×10^{-4} M are shown in Figs. 1A and 1B respectively. Maximum TPI and PA responses were observed with 1×10^{-4} M ACh, which produced a 34 per cent increase in TPI breakdown (Fig. 1A) and a 104 per cent increase in PA labeling (Fig. 1B). Higher concentrations of ACh did not produce further increases in either TPI breakdown or PA labeling. Similarly, the dose–tension relationships for ACh, carbachol and acetyl-β-methacholine were investigated (Fig. 2). Concentrations of these agonists between 1×10^{-7} M and 1×10^{-3} M caused a concentration-dependent contraction of the iris muscle.

The potencies of these agonists were determined based on doses which produced an equal biochemical or pharmacological response. In the present studies, the ED_{50} value for each agonist was taken as a measure of its potency. The ED_{50} values for the various muscarinic agonists were determined from the dose–response curves in Figs. 1 (A and B) and 2 and are summarized in

Table 1. Relative potencies (ED_{50}) of cholinergic agonists for iris muscle muscarinic receptors obtained from the biochemical and pharmacological methods described in text *

	ED_{50} (M)		
	Biochemical method		Pharmacological method†
Agonist	TPI breakdown	PA labeling	Muscle contraction
Acetylcholine	6.0×10^{-6}	1.2×10^{-6}	7.6×10^{-6}
Acetyl-β-methacholine	1.3×10^{-5}	1.5×10^{-5}	3.5×10^{-6}
Carbachol	7.0×10^{-5}	3.3×10^{-5}	1.5×10^{-6}

* These relative potency values were calculated from Figs. 1 and 2 respectively.
† In contrast to the biochemical method, eserine was omitted from the incubation medium in these experiments.

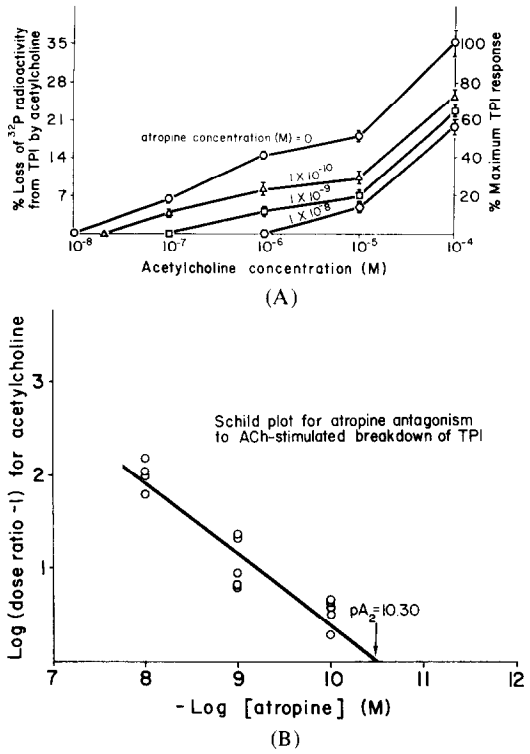


Fig. 3. Panel A: Dose–response curves (TPI breakdown) to ACh in the absence and presence of atropine in the rabbit iris muscle. Experimental conditions are the same as those described for Fig. 1A. In those experiments where the effect of atropine was studied, the blocker was added 5 min before the addition of ACh. The TPI response was expressed either as percent of the maximal TPI decrease in ^{32}P labeling or as percent decrease of ^{32}P labeling in TPI from control. Each point represents the mean of 3–5 separate determinations ± S.E.M. Panel B: Schild plot for atropine antagonism to ACh-stimulated breakdown of TPI. Data for the Schild plot were taken from the experiments in Fig. 3A.

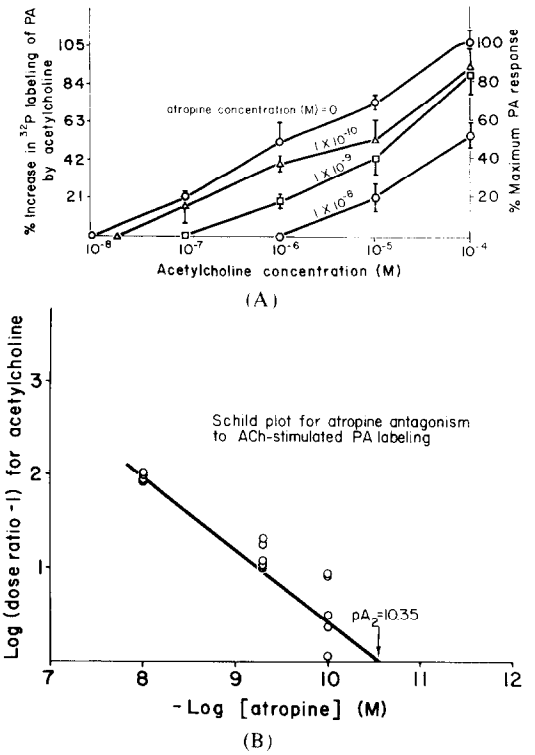


Fig. 4. Panel A: Dose–response curves (PA labeling) to ACh in the absence and presence of atropine in the rabbit iris muscle. Experimental conditions are the same as those described in the legend of Fig. 1A. In those experiments containing atropine, the blocker was added 5 min before the addition of ACh. The PA response was expressed either as per cent of the maximal increase in ^{32}P labeling in PA or as per cent increase in ^{32}P labeling of PA over the control. Each point represents the mean of 3–5 separate determinations ± S.E.M. Panel B: Schild plot for atropine antagonism to ACh-stimulated PA labeling. Data for the Schild plot were taken from the experiments in Fig. 4A.

Table 1. The affinity obtained with the pharmacological method for ACh is slightly lower than that obtained with the biochemical method (Table 1). This could be due to the fact that eserine was omitted from the incubation mixture in the former. In contrast, the affinities for acetyl-β-methacholine and carbachol obtained with the pharmacological method are higher than those obtained with the biochemical method (Table 1).

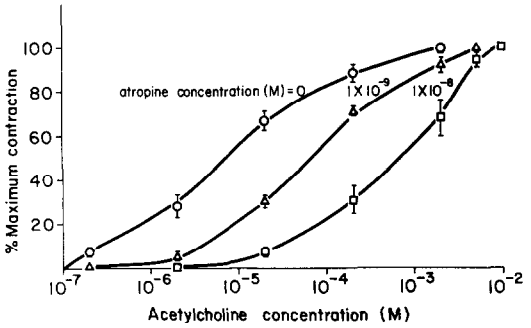
Dose–response curves (TPI breakdown, PA labeling and contraction responses) to ACh in the presence and absence of atropine and measurement of atropine antagonism

To determine the inhibition constant of atropine and to characterize the type of inhibition, the effects of a range of atropine concentrations between 1×10^{-12} M and 5×10^{-5} M were investigated. It was found that at these concentrations, atropine, in the absence of any added agonist, had no effect on either TPI breakdown or PA labeling. The sensitivities of the test systems (both biochemical and pharmacological) were reduced by addition of increasing concentrations of atropine to the incubation medium (Figs. 3A, 4A, and 5A). With concentrations of atropine of 1×10^{-8} M, 1×10^{-9} M and 1×10^{-10} M, the ACh-stimulated breakdown of TPI and PA labeling were inhibited (Figs. 3A and 4A respectively), but it was found that higher concentrations of ACh could partially overcome this blocking activity of atropine. As expected, increasing concentrations of atropine progressively shifted the dose–TPI and dose–PA response curves to the right in a near parallel fashion (Figs. 3A and 4A respectively). Similarly, in the presence of 1×10^{-8} M and 1×10^{-9} M atropine, the dose–tension curve to ACh was shifted to the right in a parallel fashion (Fig. 5A).

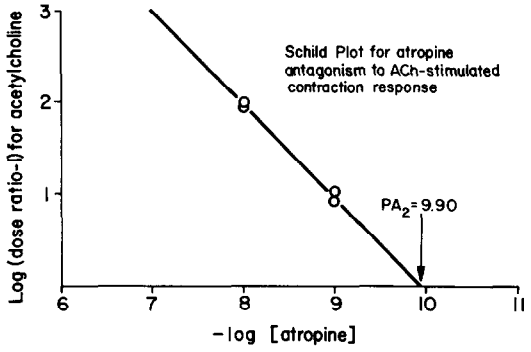
Table 2. Dissociation constants for the atropine–muscarinic receptor complex obtained from the biochemical* and pharmacological† methods described in text.

Method of determination	pA_2	$-\log K_B$
TPI response	10.30	9.76 (15)‡
PA response	10.35	8.70 (22)
Contraction response	9.90	9.94 (6)

* Calculated from Figs. 3A and B and 4A and B.
† Calculated from Figs. 5A and B.
‡ Total number of individual determinations.



(A)



(B)

Fig. 5. Panel A: Dose-response curves (contraction) to ACh in the absence and presence of atropine in rabbit iris muscle. Experimental conditions were the same as those described in Materials and Methods. Control responses to ACh only were determined first. The muscles were then washed, allowed to re-equilibrate, and atropine was added. The tissues were incubated for an additional 60 min in the presence of atropine before the addition of ACh. Isometric contractions to ACh stimulation were recorded, and response was plotted as per cent of the maximum recorded contraction to ACh. Each point represents the mean ± S.E.M. of eight individual determinations. Panel B: Schild plot for atropine antagonism to ACh-stimulated contraction response. Data for the Schild plot were taken from the experiments in Fig. 5A.

In order to ascertain that the inhibitory effect of atropine (Figs. 3A, 4A and 5A) did involve a competitive reversible inhibition, according to receptor theory, and to allow for calculation of the dissociation constant for the receptor–antagonist complex K_B, the log dose ratio -1 was plotted as a function of the negative logarithm of the atropine concentration (Figs. 3B, 4B and 5B) according to Arunlakshana and Schild [9]. The points where these plots intercept the abscissae (at log dose ratio $-1 = 0$) are defined as the pA_2 values (Figs. 3B, 4B and 5B). The slopes obtained from the Schild plots in Figs. 3B, 4B and 5B were found to be -0.80, -0.85 and -1.03, respectively. These values are sufficiently near unity to be consistent with the antagonist acting in a competitive manner with ACh for the receptor. The calculated K_B values (see Materials and Methods) and the corresponding pA_2 values are summarized in Table 2.

DISCUSSION

The problem to which we have addressed ourselves in the present work is whether the observed effects of ACh on TPI metabolism in the rabbit iris smooth muscle, which can occur at physiological concentrations and are antagonized by atropine [1–6], are in some way related to the contraction process, which is known to be initiated by the interaction of ACh with its cholinergic muscarinic receptor. An increasing number of comparisons of kinetic constants determined by biochemical and pharmacological procedures are becoming available, and the agreement among the constants obtained by the two methods is quite good (Table 3). As with many of the biochemical responses, little information has been available regarding the dose–response curves for phosphoinositide turnover [17, 18]. Hopefully, the dose–response curves for cholinergic agonist stimulation of TPI breakdown and PA labeling of the iris reported in this communication will fill the gap. In addition, these studies enabled us to make quantitative comparisons between these biochemical responses and the pharmacological response. Cholinergic agonist-induced TPI breakdown and PA labeling were found to respond in a dose-dependent manner (Fig. 1A and B), which correlates well with the dose–tension curves (Fig. 2) obtained from the pharmacological procedure. Thus, when the agonist was ACh, the kinetic constants obtained for guinea pig ileum from the radio-ligand binding method and for rabbit iris from the pharmacological and biochemical methods (ED_{50} are comparable, being of the order of $1–8 \times 10^{-6}$ M (Table 3). Similarly, the kinetic constants obtained in the presence of the cholinergic agonists, carbachol and acetyl-β-methacholine, by the above procedures are quite good, there being no more than a few-fold difference in the case of either drug (Table 3).

Atropine has been shown previously to be a competitive antagonist at muscarinic receptors [9], and therefore evidence was sought for competition against ACh in this muscle. For measurement of antagonism, the effects of atropine on dose–TPI, –PA and –contraction responses to ACh were studied. Atropine, at concentrations between 1×10^{-10} M and 1×10^{-8} M, produced a parallel shift to the right in the ACh dose–response curves (Figs. 3A, 4A and 5A). In contrast to the pharmacological response, where atropine inhibition of ACh-induced contraction was overwhelmed by concentrations of ACh of $1 \times 10^{-3}–1 \times 10^{-2}$ M, atropine inhibition of ACh-stimulated breakdown of TPI and PA labeling was only partially reversed by 10^{-4} M ACh (Figs. 3A and 4A). This could be due to the fact that ACh at high concentrations ($1 \times 10^{-3}–1 \times 10^{-2}$ M) exerts an inhibitory effect on TPI breakdown and PA labeling (M. J. Grimes and A. A. Abdel-Latif, unpublished observations). Furthermore, it is well established that in the case of many reversible competitive antagonists, a steady-state level of blockade is not achieved unless one has allowed the tissue to be in contact with the antagonist for a long period of time (60 min for the pharmacological response, Fig. 5A). In the biochemical procedure, waiting for such long periods will bring about more than 90 per cent loss in ^{32}P-radioactivity from TPI. Since the pA_2 and K_B values may be obtained before a final steady-state level of blockade by atropine has been reached, these kinetic constants are a measure of the "apparent" dissociation constant for the atropine antagonism of the ACh-stimulated responses [19]. When these inhibitory effects of atropine were tested for competitive antagonism in the form of a Schild plot for

Table 3. Comparison of kinetic constants derived from binding, contraction and biochemical responses in smooth muscle

Ligand	Method	Preparation	Kinetic constants: Binding (M)	Kinetic constants: ED_{50} (M)	Kinetic constants: K_B (M)	pA_2	Ref.
Acetylcholine	ID_{50} against [^{3}H] QNB‡ binding	guinea pig ileum	$2–4 \times 10^{-6}$				10
	Contraction	Bovine sphincter		3.8×10^{-4}			11
	Contraction	Guinea pig ileum		5.8×10^{-8}			12
	Contraction	Brain vessels		1.76×10^{-6}			13
	Contraction	Rabbit iris		7.6×10^{-6}			*
	TPI	Rabbit iris		1.2×10^{-6}			*
	PA	Rabbit iris		6.0×10^{-6}			*
Carbachol	ID_{50} against binding of [^{3}H]QNB	guinea pig ileum	$2–3 \times 10^{-5}$				10
	Contraction	Bovine sphincter		9.3×10^{-8}			11
	Contraction	Guinea pig ileum		$0.5–3 \times 10^{-7}$			12
	Contraction	Guinea pig ileum		1.1×10^{-7}			14
	PI breakdown§	Guinea pig ileum		1.0×10^{-5}			15
	Contraction	Rabbit iris		1.5×10^{-6}			*
	TPI	Rabbit iris		7.0×10^{-5}			*
	PA	Rabbit iris		3.3×10^{-5}			*
Acetyl-β-methacholine	ID_{50} against binding of [^{3}H]QNB	Guinea pig ileum	$3–5 \times 10^{-6}$				10
	Contraction	Bovine sphincter		5.2×10^{-6}			11
	Contraction	Guinea pig ileum		3.3×10^{-8}			14
	Contraction	Rabbit iris		3.5×10^{-6}			*
	TPI	Rabbit iris		1.3×10^{-5}			*
	PA	Rabbit iris		1.5×10^{-5}			*
Acetylcholine–atropine antagonism	Contraction	Guinea pig ileum			1×10^{-9}	8.6; 8.4	9, 16
	Contraction	Brain vessels			1×10^{-10}	10.1	13
	Contraction	Rabbit iris			1.14×10^{-10}	9.9	†
	TPI	Rabbit iris			1.7×10^{-10}	10.3	†
	PA	Rabbit iris			2×10^{-9}	10.35	†

* Table 1 in the present study.
† Table 2 in the present study.
‡ [^{3}H]quinuclidinyl benzilate.
§ PI = phosphatidyl imositol.

the TPI, PA and contraction responses, straight lines with slopes near unity were obtained (Figs. 3B, 4B and 5B). The fact that the slopes for the TPI– and PA–Schild plots (Figs. 3B and 4B respectively) were lower than that obtained for the contraction–Schild plot (Fig. 5B) could be due to the inability to reverse the atropine inhibition completely in the biochemical response (see Discussion above). The data presented suggest that atropine produced a parallel shift in the dose–TPI, –PA and –contraction response curves by competing with ACh for the receptor site. The findings of similar, although not identical, K_B values for the atropine–muscarinic receptor, along with similar pA_2 values from the Schild plots, with the TPI, PA and contraction responses in the rabbit iris (Table 3) could suggest either of the following relationships between the biochemical and pharmacological responses (equation 1):

$$\text{ACh} + \text{R} \xrightarrow{\quad} \text{ACh–R complex} \quad (a)$$

$$\rightarrow Ca^{2+} \text{ influx} \uparrow \quad \begin{cases} \nearrow \text{TPI breakdown} \rightarrow \text{Muscle contraction} & (b) \\ \searrow \text{Muscle contraction} \rightarrow \text{TPI breakdown} & (b) \end{cases} \quad (1)$$

Thus, in this tissue, TPI breakdown may be involved in

the chain of events leading from cholinergic muscarinic stimulation to muscle response (equation 1, a) or it may follow muscle contraction (equation 1, b).

In the present study, we have followed the basic assumption that similarities in the characteristics of different responses could reflect similarities in the receptor(s) mediating these responses [20]. Although suggestions have been made and some evidence has been obtained that the molecular mechanism underlying the stimulus–transfer chain linking receptor occupancy with response may involve changes in membrane permeability, PI turnover, cyclic GMP levels and cation fluxes [21, 22], the chain of events leading from cholinergic muscarinic stimulation to muscle response is still unclear. The data presented in this paper strongly suggest that in smooth muscle phosphoinositide metabolism could be involved in muscle contraction.

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