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Dual Pathways in Muscarinic Receptor Stimulation of Phosphoinositide Hydrolysis

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ABSTRACT: The relationships between phosphoinositide hydrolysis induced by various muscarinic agonists and by membrane depolarization agents were investigated in rat cerebral cortex and heart atrium slices. In both preparations, phosphoinositide hydrolysis was stimulated by a combination of carbamylcholine and membrane depolarization with 40 mM K^+ in a synergistic fashion. The synergism was more pronounced at lower external calcium ion concentrations and was sensitive to verapamil. Lower external calcium ion concentrations were required for demonstration of the synergism in heart atrium slices than in cerebral cortex slices. The carbamylcholine-induced stimulation was only partially additive with membrane depolarization via Na^+ channel gating by batrachotoxin. In addition, K^+ depolarization eliminated the sensitivity of carbamylcholine-stimulated phosphoinositide hydrolysis to the sodium channel blocker tetrodotoxin. Our results suggest that muscarinically stimulated phosphoinositide hydrolysis in rat cerebral cortex and heart atrium slices may occur by dual pathways which interact synergistically and that only one of the pathways is depolarization-dependent. Different muscarinic agonists could preferentially utilize these pathways, thus perhaps explaining their different potencies in stimulating phosphoinositide hydrolysis.

Activation of cholinergic muscarinic receptors in various tissues leads to increased hydrolysis of membrane phosphoinositides (Berridge & Irvine, 1984; Hokin, 1985). Inositol 1,4,5-trisphosphate ($InsP_3$),[1] a product of phosphatidylinositol 4,5-bisphosphate hydrolysis, functions in many instances as a second messenger for mobilization of intracellular Ca^{2+} stores, which in turn mediate the physiological response to muscarinic stimulation (Berridge & Irvine, 1984; Hokin, 1985; Berridge et al., 1982, 1983; Aub & Putney, 1985). Another product of phosphoinositide hydrolysis is diacylglycerol (DG), which activates protein kinase C (PK-C) (Kikkawa et al., 1982; Nishizuka, 1984). This enzyme is also activated by phorbol esters, which block phosphoinositide hydrolysis by activated muscarinic (Orellana et al., 1985; Vicentini et al., 1985) and other receptors (Leeb-Lundberg et al., 1985). We have recently described interactions between muscarinic receptors and the Na^+ channel ligand batrachotoxin (BTX) (Cohen-Armon et al., 1985; Cohen-Armon & Sokolovsky, 1986). Possible involvement of a sodium channel gating mechanism (or mechanisms) in the muscarinic stimulation of phosphoinositide hydrolysis and in the different potencies observed for various muscarinic agonists (Fisher & Agranoff, 1981; Brown & Brown, 1984; Brown-Masters et al., 1984; Brown et al., 1984; Fisher et al., 1984; Gonzales & Crews, 1984; Jacobson et al., 1985) is therefore implicated.

Membrane depolarization by high extracellular $[K^+]$ induces phosphoinositide hydrolysis in several neuronal tissues (Kendall

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[1] Abbreviations: BTX, batrachotoxin; TTX, tetrodotoxin; $[Ca^{2+}]_i$, intracellular calcium ion concentration; $[Ca^{2+}]_o$, extracellular calcium ion concentration; PMA, phorbol 12-myristate 13-acetate; InsP, inositol 1-phosphate; $InsP_3$, inositol 1,4,5-trisphosphate; DG, diacylglycerol; PL-C, phospholipase C; PK-C, protein kinase C; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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& Nahorski, 1984; Bone & Michell, 1985; Gusovsky et al., 1986), possibly through the activation of phospholipase C (PL-C) by elevated intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) (Hirasawa et al., 1982; Hofmann & Majerus, 1982; Siess & Lapetina, 1983; Wilson et al., 1984, 1985). PL-C requires higher Ca^{2+} concentrations for the hydrolysis of phosphatidylinositol than of phosphoinositides (Wilson et al., 1984). Since the former is the major plasma membrane phosphoinositide, elevated $[Ca^{2+}]_i$ may substantially increase the formation of inositol 1-phosphate (InsP), with only minor effects on $InsP_3$ formation. Indeed, elevation of $[Ca^{2+}]_i$ by the Ca^{2+} ionophore A23187 increases phosphoinositide hydrolysis in synaptosomes (Fisher & Agranoff, 1981) and in cerebral cortex slices (Kendall & Nahorski, 1984).

A similar pathway, i.e., depolarization-induced elevation of $[Ca^{2+}]_i$ resulting in enhancement of phosphatidylinositol cleavage by PL-C, may also be functioning in muscarinic receptor mediated [^{3}H]InsP accumulation in certain tissues. While for some tissues it is widely accepted that activation of muscarinic receptors leads to a mobilization of intracellular Ca^{2+} via $InsP_3$, there is some controversy as to whether this takes place in other tissues (Brown & Brown, 1983; Brown et al., 1985); it was tentatively suggested that metabolic pathways arising from the phosphoinositide-derived DG may be the ones mediating the physiological response (Brown & Brown-Masters, 1984). This possibility may be especially relevant in the case of muscarinic receptors stimulated phosphoinositide hydrolysis in the brain, where chelation of extracellular Ca^{2+} prevents [^{3}H]InsP accumulation (Gonzales & Crews, 1984; Kendall & Nahorski, 1984).

In the present study the possible involvement of a Na^+ channel gating mechanism (or mechanisms) and extracellular Ca^{2+} in the muscarinic stimulation of phosphoinositide hydrolysis was investigated in slices of rat cerebral cortex, the brain region in which the muscarinic stimulation of phosphoinositide hydrolysis is most pronounced (Gonzales & Crews, 1984). In view of the possible involvement of guanine nucleotide binding proteins in receptor-mediated phosphoinositide hydrolysis (Cockcroft & Gompers, 1985; Smith et al., 1986), parallel studies were performed in rat atrium slices, in which the interactions between muscarinic receptors and guanine nucleotide binding proteins are reportedly different from those encountered in the cerebral cortex (Gurwitz et al., 1985).

Experimental Procedures

Materials. Muscarinic ligands and other drugs were from Sigma, except for batrachotoxin, which was the generous gift of Dr. J. W. Daly, National Institutes of Health, Bethesda, MD. [^{3}H]Inositol (17 Ci/mmol) was from Amersham International PK, Amersham, U.K.

Assay for Phosphoinositide Hydrolysis. The protocol outlined by Gonzales and Crews (1984) was followed. Briefly, adult male rat cerebral cortex or heart atrium slices (200 × 200 μm) were cut with a Sorvall tissue chopper and dispersed in a buffer (composition, mM: NaCl, 123; KCl, 5; KH_2PO_4, 1.3; $CaCl_2$, 0.8; glucose, 10; HEPES, 20, pH 7.4 at 37 °C) prewarmed to 37 °C and oxygenated. Following three incubations with buffer changes every 10 min, 5 μCi/mL [^{3}H]-inositol (17 Ci/mmol; Amersham International PK, Amersham, U.K.) was added and the slices were incubated for 60 min; this was followed by four washes with fresh buffer replaced every 10 min. In some experiments calcium was omited from the washing buffer, as indicated in the figures. The slices were then allowed to settle by gravity, and 50-μL aliquots were gently transferred to 5-mL Biovials containing 200 μL of the same buffer with the addition of 10 mM LiCl (final concentration, isotonically substituted for NaCl) and the indicated additions (all at final concentrations). The calcium concentration was 0.8 mM, except where noted otherwise. The vials were oxygenated, capped tightly, and incubated for 20 min at 37 °C. Reactions were stopped by adding 1 mL of chloroform–methanol (1:2). Extraction, separation of aqueous and chloroform phases, and separation of the water-soluble products of phosphoinositide hydrolysis by ion-exchange chromatography were carried out by the method of Berridge et al. (1982). Samples of the chloroform phase were dried and counted in parallel with the InsP fraction eluted from the columns. This enabled results to be expressed as the ratio of dpm in the InsP fraction to total dpm incorporated (InsP fraction plus chloroform samples, corrected for sample volume), thereby significantly reducing interassay as well as intraassay variations (Gonzales & Crews, 1984). The control value for [^{3}H]InsP accumulation in LiCl-containing buffer with no additions was subtracted from the values thus determined; for example, in the experiment depicted in Figure 1, the recorded control value of 2.1 dpm/100 dpm incorporated represents 156 dpm detected in the InsP fraction and 7208 dpm detected in the chloroform phase (following correction for sample volume). For K^+ depolarization, 40 mM KCl was isotonically substituted for NaCl. The indicated compounds were added simultaneously upon transfer of the slices to the Li^+-containing buffer, except for tetrodotoxin (TTX), which was already present during the 10 min of the last was preceding this step. Incubation periods of 20 min were chosen since preliminary experiments had shown low, inaccurate signals for shorter incubations and deviations from linearity for incubations exceeding 30 min. Atropine (20 μM) but not hexamethonium could block the response to carbamylcholine under all situations; thus, the response to carbamylcholine is muscarinic, in agreement with previous reports (Fisher & Agranoff, 1981; Brown & Brown, 1984; Brown-Masters et al., 1984; Brown et al., 1984; Fisher et al., 1984; Gonzales & Crews, 1984; Jacobson et al., 1985). None of these cholinergic antagonists affected the responses to K^+ or BTX depolarization. Saturating concentrations of agonists were required in order to obtain accurate data. Thus, results are presented for 3, 1, and 0.1 mM carbamylcholine, arecoline, and oxotremorine, respectively; higher concentrations of these agonists did not result in more extensive accumulation of [^{3}H]InsP under any of the experimental conditions tested.

Results and Discussion

Muscarinic agonists enhance [^{3}H]BTX binding and hence the opening of Na^+ channels (Cohen-Armon & Sokolovsky, 1986); they may therefore promote phosphoinositide hydrolysis by elevating $[Ca^{2+}]_i$. The data in Figures 1 and 2 indicate that this indeed occurs in rat cerebral cortex slices. Both K^+ depolarization and BTX-induced depolarization increased phosphoinositide hydrolysis in a Ca^{2+}-dependent fashion. The effects of these depolarizing agents were not additive (Figure 1), implying that they act on the same cell population and share (at least partially) the same pathway. However, only the BTX-induced effect was sensitive to TTX. This implies that it is not the opening of the voltage-sensitive Na^+ channel that is responsible for the increase in phosphoinositide hydrolysis but rather that a more distal common site of action is involved. A likely candidate is the voltage-sensitive Ca^{2+} channel, since its blockade by 10 μM verapamil (Figure 2) eliminates the depolarization-induced phosphoinostide hydrolysis. Note that verapamil was more effective at lower concentrations of extracellular Ca^{2+}, $[Ca^{2+}]_o$; this supports the

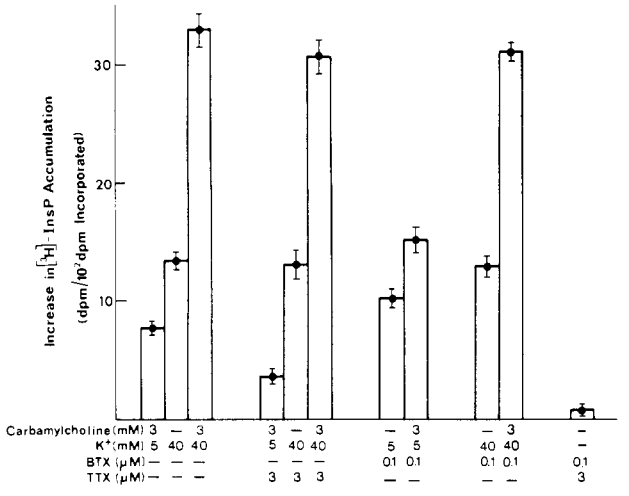

FIGURE 1: Effects of combinations of carbamylcholine, K^+ depolarization, batrachotoxin (BTX), and tetrodotoxin (TTX) on phosphoinositide hydrolysis in rat cerebral cortex slices. Data are means ± SEM of a typical experiment carried out in triplicate.

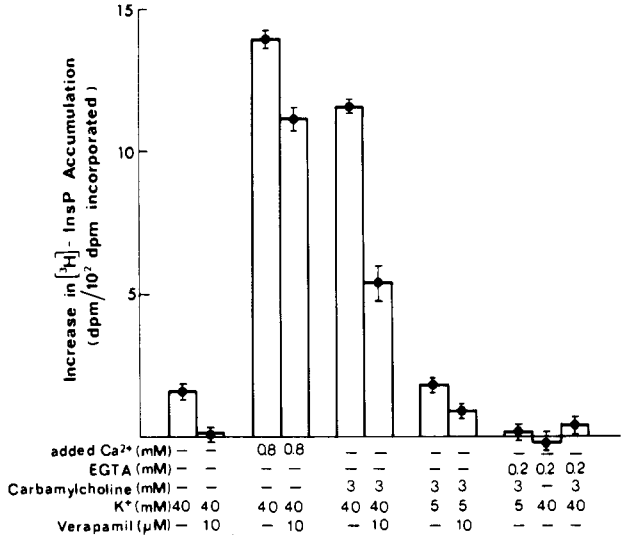

FIGURE 2: Blockade by verapamil and by EGTA of carbamylcholine-induced and K^+ depolarization induced increase in phosphoinositide hydrolysis. Note that in the absence of externally added Ca^{2+} a concentration of 11 μM was recorded in the same preparation (Kendall & Nahorski, 1984). The buffer used to wash slices following the labeling stage did not include $CaCl_2$, and the assay buffer included the indicated concentrations of added $CaCl_2$. Data are means ± SEM of a typical experiment carried out in triplicate.

interpretation that it is antagonism of Ca^{2+} by verapamil at Ca^{2+} channels which blocks the depolarization-induced effect (Figure 2).

In addition, neither the K^+-induced nor the BTX-induced depolarization could elevate [^{3}H]InsP in a buffer containing 200 μM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) in the absence of externally added calcium (Figure 2). Elevation of $[Ca^{2+}]_i$ with the ionophore A23187 increased [^{3}H]InsP accumulation (e.g., 5.6 ± 0.8 dpm/100 dpm incorporated at 1 μg/mL A23187 with 0.8 mM added Ca^{2+}), confirming a previously reported finding (Kendall & Nahorski, 1984). These data imply that hydrolysis of phosphoinositides may result from Ca^{2+} influx mediated by either K^+- or BTX-induced depolarization in rat cerebral cortex slices.

In order to determine whether a similar pathway functions in muscarinic receptor mediated phosphoinositide hydrolysis, carbamylcholine was employed in various combinations with the two depolarizing agents, Na^+ channel and Ca^{2+} channel

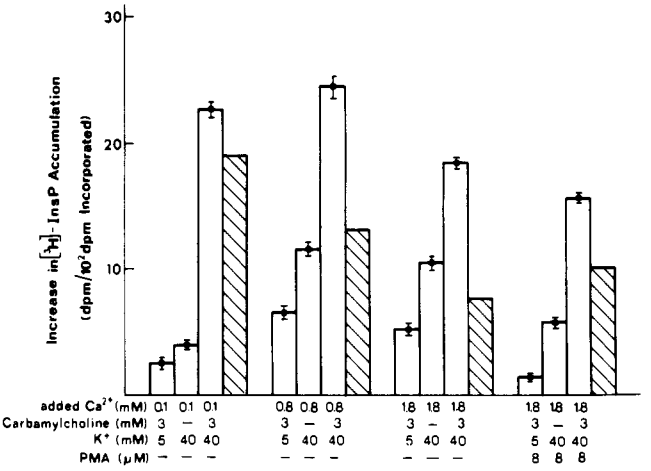

FIGURE 3: Calcium dependency of carbamylcholine-induced and K^+ depolarization induced increases in phosphoinositide hydrolysis, and its inhibition by phorbol 12-myristate 13-acetate (PMA). Shaded bars represent the carbamylcholine-induced increase in [^{3}H]InsP accumulation beyond the stimulation recorded with 40 mM KCl.

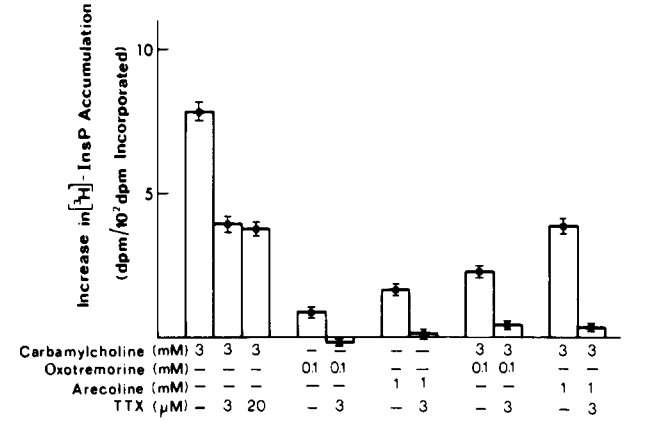

FIGURE 4: Potency of various muscarinic agonists in stimulating phosphoinositide hydrolysis and their sensitivity to TTX.

blockers, and varying concentrations of Ca^{2+} (Figures 1–3). Carbamylcholine-induced [^{3}H]InsP accumulation was partially sensitive to TTX (Figure 1), indicating the involvement of a depolarization-related mechanism like the one mediating BTX-stimulated phosphoinositide hydrolysis. The effects of BTX and carbamylcholine were partially additive (Figure 1), suggesting that they share—at least partially—a common pathway, namely Na^+ channel gating. Verapamil sensitivity of the response to carbamylcholine in the presence of 40 mM K^+ again shows the involvement of voltage-dependent Ca^{2+} channels (Figure 2). Similar results were obtained under the same conditions with other Ca^{2+} channel blockers, nifedipine and nimodipine (1 μM), which inhibited the increase in [^{3}H]InsP accumulation by 45% and 37%, respectively. However, there was an additional component of carbamylcholine-stimulated phosphoinositide hydrolysis that was resistant even to 20 μM TTX (Figure 4), indicating the involvement of a second, Na^+ channel independent pathway. Further evidence for such a pathway comes from the ability of carbamylcholine to increase [^{3}H]InsP accumulation beyond the capacity of BTX to do so. The high concentration of carbamylcholine employed (3 mM) excludes the possibility that interconversions of muscarinic receptors between affinity states (Gurwitz et al., 1985) or changes in affinity constants (Cohen-Armon et al., 1985; Cohen-Armon & Sokolovsky, 1986) may have been involved in the phenomena described. Thus, the higher [^{3}H]InsP accumulation observed with car-

bamylcholine and BTX (than with carbamylcholine alone) cannot be explained by an increased agonist affinity induced by BTX at the muscarinic receptor (Cohen-Armon et al., 1985; Cohen-Armon & Sokolovsky, 1986). Nor can the phenomena be attributed to either higher-affinity or low-affinity agonist binding sites (Gurwitz et al., 1985), because of the high agonist concentrations required to obtain accurate measurements of [^{3}H]InsP accumulation (see Experimental Procedures).

The TTX-insensitive component predominated when carbamylcholine-stimulated phosphoinositide hydrolysis was measured under conditions of K^+ depolarization (Figure 1). Moreover, the muscarinic response was more marked in this case; i.e., there was synergism between the effects of carbamylcholine and K^+ depolarization (Figure 1). This synergism was very pronounced at low $[Ca^{2+}]_o$ concentrations but was rather weak at $[Ca^{2+}]_o$ = 1.8 mM (Figure 3); thus the TTX-insensitive pathway of carbamylcholine-stimulated phosphoinositide hydrolysis is inversely dependent on $[Ca^{2+}]_o$. This is in contrast with the positive correlation found between $[Ca^{2+}]_o$ and the phosphoinositide hydrolysis induced by either K^+ or BTX depolarization. The carbamylcholine-stimulated accumulation of [^{3}H]InsP was also increased in a TTX-insensitive manner by the presence of A23187 (e.g., from 7.3 ± 0.3 to 17.1 ± 0.7 dpm/100 dpm incorporated in the absence and presence of 1 μg/mL A23187, respectively, with the addition of 0.8 mM Ca^{2+}). This supplements earlier observations of carbamylcholine-stimulated phosphatidate labeling in synaptosomes (Fisher & Agranoff, 1981).

A dual pathway may explain why some muscarinic agonists, e.g., oxotremorine and arecoline, are less effective than others in stimulating phosphoinositide hydrolysis (Fisher & Agranoff, 1981; Brown & Brown, 1984; Brown-Masters et al., 1984; Brown et al., 1984; Fisher et al., 1984; Gonzales & Crews, 1984; Jacobson et al., 1985). The more pronounced TTX sensitivity of the oxotremorine- and arecoline-stimulted [^{3}H]InsP accumulation (Figure 4) indicates that the Na^+ channel pathway is the more important one for their action. They are therefore capable of decreasing the potency of carbamylcholine (Brown & Brown, 1984; Gonzales & Crews, 1984; Jacobson et al., 1985) (Figure 4) through competition. The residual response to carbamylcholine in the presence of, e.g., oxotremorine was indeed highly sensitive to TTX (Figure 4). The weak ability of the less efficacious agonists to utilize the TTX-insensitive pathway was also reflected by their limited potency in K^+ depolarization buffer (Figure 5). The latter suggestion is compatible with the ability of oxotremorine and arecoline to increase the binding of [^{3}H]BTX (Cohen-Armon & Sokolovsky, 1986).

The results presented above suggest the existence of a pathway that leads to increased phosphoinositide turnover in response to muscarinic stimulation and that involves depolarization induced through voltage-sensitive Na^+ channels. The depolarization elevates $[Ca^{2+}]_i$, leading to increased phosphoinositide hydrolysis [presumably by activating PL-C (Hirasawa et al., 1982; Hofmann & Majerus, 1982; Siess & Lapetina, 1983; Wilson et al., 1984, 1985)] and the production of DG. However, the existence of an additional pathway, which does not involved voltage-sensitive Na^+ channels, is suggested by the inability of TTX to completely block the muscarinic stimulation and by the synergism between stimulation by K^+ depolarizaton and by the muscarinic receptors. The question then arises: what are the phenomena that bring this synergism? One attractive possibility would involve the conversion of protein kinase C (PK-C) from an active to an inactive form by agonist-occupied muscarinic receptors in the

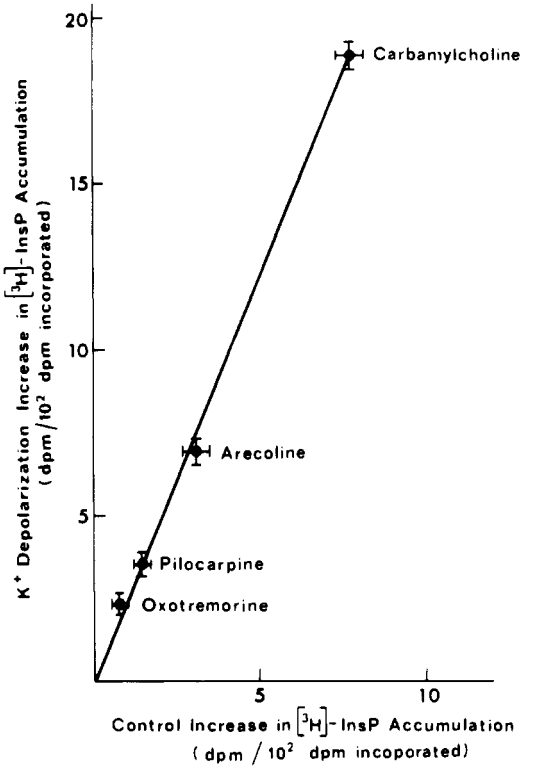

FIGURE 5: Relationship between potency of various agonists in stimulating phosphoinositide hydrolysis in control buffer and their potency in K^+ depolarization buffer. Ordinate values represent the increase *beyond* the stimulation recorded with 40 mM KCl. Calcium was 0.8 mM.

TTX-insensitive pathway. Such a conversion could occur directly or indirectly, e.g., by translocation of PK-C to a cytosolic pool, as reported for the action of adrenocorticotropin (ACTH) in adrenocortical cells (Vilgrain et al., 1984); alternatively, translocation of the muscarinic receptors themselves, as proposed recently (Harden et al., 1985), could render them less accessible to PK-C. Since activated PK-C inhibits phosphoinositide hydrolysis (Orellana et al., 1985; Vicentini et al., 1985; Leeb-Lundberg et al., 1985), this second pathway would be more effective under conditions where a high proportion of PK-C is in the active state. This proposal would offer an explanation of the synergistic activation of phosphoinositide hydrolysis by K^+ depolarization and carbamylcholine: under depolarization conditions, a higher proportion of PK-C is in the active form, and suppression of the feedback inhibition by carbamylcholine is therefore more pronounced. This explanation is also in line with the inhibition of phosphoinositide hydrolysis by phorbol 12-myristate 13-acetate (PMA): this phorbol ester activates PK-C and should therefore inhibit the increased phosphoinositide hydrolysis by both pathways, although to differing extents. Such indeed was found to be the case (Figure 3). It should be noted that inhibition of carbamylcholine-stimulated phosphoinositide hydrolysis by PMA was undetectable below 1 μM PMA (data not shown). This is in contrast with results obtained in astrocytoma cells (Orellana et al., 1985), where total inhibition was observed at 0.1 μM PMA. The reasons for this discrepancy are unclear. The synergism appears to be stronger at lower $[Ca^{2+}]_o$ concentrations (Figure 3): such a situation could arise if the dependence of PL-C activity on $[Ca^{2+}]_o$ is stronger than that of PK-C.

In view of the cellular heterogeneity of cerebral cortex slices, we cannot exclude the possibility that each of the dual pathways described above functions in distinct cell populations. In

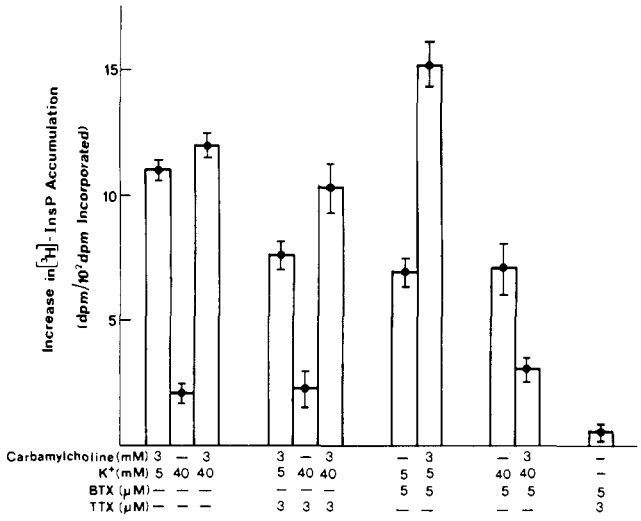

FIGURE 6: Effects of combinations of carbamylcholine, K^+ depolarization, batrachotoxin (BTX), and tetrodotoxin (TTX) on phosphoinositide hydrolysis in rat atrial slices. Data are means ± SEM of a typical experiment carried out in triplicate.

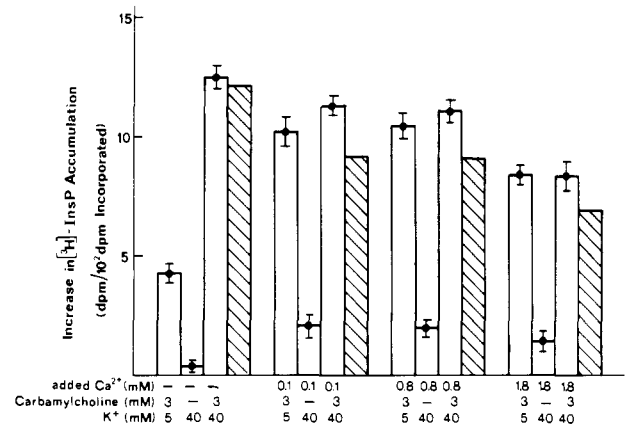

FIGURE 7: Calcium dependency of carbamylcholine-induced and K^+ depolarization induced increases in phosphoinositide hydrolysis in rat atrial slices. See Figures 2 and 3 for more details.

this case, the synergism would arise from specific interactions between different cell types. However, in such a case one would expect to observe synergism between BTX and carbamylcholine, since BTX acts through depolarization (as does K^+). This did not occur (Figure 1), thus weakening the above possibility.

To further elucidate the mechanism of the muscarinic stimulation of phosphoinositide hydrolysis and the involvement of depolarization-related phenomena, we carried out parallel studies in slices of rat atrium. This preparation was chosen mainly for two reasons: (i) In the heart the muscarinic receptors are located mainly on myocytes, and complexities arising from possible involvement of interneurons in depolarization-related phenomena are therefore avoided. (ii) Previous studies have indicated that the interaction between the muscarinic receptor–agonist complex and guanine nucleotide binding protein(s) are different in the cerebral cortex and the heart (Gurwitz et al., 1985). Since the involvement of guanine nucleotide binding proteins was implicated in receptor-mediated phosphoinositide hydrolysis (Cockcroft & Gompers, 1985; Smith et al., 1986), as well as in the gating of Na^+ channels (Cohen-Armon & Sokolovsky, 1986), a comparison between the muscarinically stimulated phosphoinositide hydrolysis in cerebral cortex and tat in atrium slices could provide interesting information on the pathways leading to this phenomenon in the two preparations.

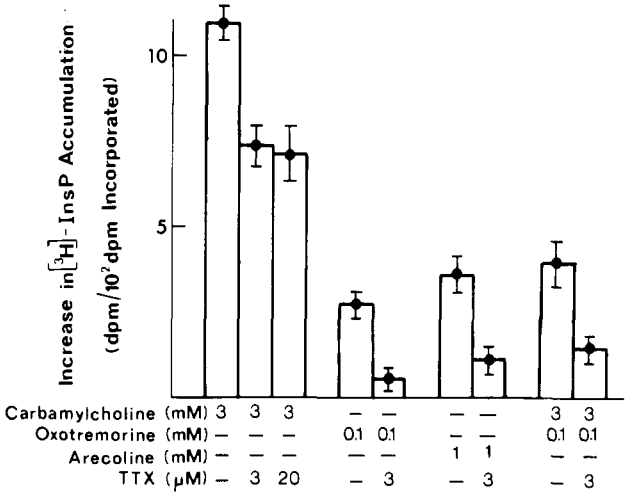

FIGURE 8: Potencies of various muscarinic agonists in stimulating phosphoinositide hydrolysis in rat atrial slices.

Data from experiments with heart atrial preparation, which were carried out similarly to those using cerebral cortex slices, are shown in Figures 6–8. As in the cerebral cortex, carbamylcholine-induced [^{3}H]InsP accumulation was partially sensitive to TTX in the atrial preparation; however, the sensitivity here was weaker than in the cerebral cortex (≈28% in the atrium vs. ≈50% in the cortex) (Figure 6). This tissue heterogeneity cannot be explained by poor accessibility of TTX to the Na^+ channels in the atrial preparation, since TTX (3 μM) could completely block the BTX-induced phosphoinositide hydrolysis (Figure 6), which is reminiscent of the situation in the cerebral cortex (Figure 1). It would thus seem that, unlike that in the cortex, the TTX-sensitive pathway in the atrial preparation is the minor one in muscarinically stimulated phosphoinositide hydrolysis.

As in the cerebral cortex, synergism between carbamylcholine and K^+ depolarization induced phosphoinositide hydrolysis was evident in the atrial slices (Figure 7); however, this synergism was observed only in medium without added calcium (i.e., about 10 μM Ca^{2+}) (Kendall & Nahorski, 1984) and disappeared when $[Ca^{2+}]_o$ was raised to 0.1 mM (Figure 7). This may be the result of different Ca^{2+} dependencies of PL-C and/or PK-C in the atrium and in the cerebral cortex, so that the "Ca^{2+} dependence" of the synergism is shifted to lower $[Ca^{2+}]_o$ concentrations. Alternatively, the differences may arise from the higher $[Ca^{2+}]_i$ concentrations (as a function of $[Ca^{2+}]_o$) in the atrial preparation than in the cerebral cortical one under our experimental conditions. The heart atrium preparation is highly sensitive to anoxia (Altschuld et al., 1985). The short period of anoxia that presumably occurs during tissues preparation would result in higher $[Ca^{2+}]_i$, which would in turn depend on $[Ca^{2+}]_o$ (Altschuld et al., 1985). More studies are required in order to clarify these possibilities; unfortunately, no established values for $[Ca^{2+}]_i$ are available for intact mammalian neurons, so comparison with the $[Ca^{2+}]_i$ levels recorded for atrial myocytes is not feasible at this stage. Moreover, even if neuronal $[Ca^{2+}]_i$ were measurable, one has to bear in mind the cellular heterogeneity of the preparations studied, as well as the possibility of subcellular compartmentalization (Yarom et al., 1985). We have therefore not attempted to correlate our data with the effects of depolarizing agents and muscarinic agonists on, e.g., $^{45}Ca^{2+}$ fluxes.

Interestingly, the [^{3}H]InsP accumulation induced by either K^+ depolarization or BTX-mediated depolarization was lower in the atrium than in the cerebral cortex (Figure 7). Yet these effects were blocked by 10 μM verapamil (in a buffer without

added Ca^{2+}; not shown), indicating that a rise in cytosolic Ca^{2+} resulting from depolarization-induced Ca^{2+} channel opening may again be involved. Thus, the smaller effect of K^+ depolarization in the atrial preparation may also result from higher $[Ca^{2+}]_i$ values for the atrial preparation, as discussed above.

The accumulation of [^{3}H]InsP induced by oxotremorine or by arecoline in the atrium was lower than the amount recorded for carbamylcholine (Figure 8). These findings are in agreement with former reports that oxotremorine and arecoline are less efficacious than carbamylcholine in atrial slices (Brown & Brown, 1983), as well as in chick myocyte cultures (Brown & Brown, 1984). The oxotremorine- and arecoline-stimulated [^{3}H]InsP accumulation could be increased by K^+ depolarization (in a buffer without added Ca^{2+}) in a manner reminiscent of the findings in the cerebral cortex. In addition, it was more sensitive to TTX (the inhibition levels recorded for the atrial preparation with 3 μM TTX in a typical experiment were 80%, 69%, and 28% of the signals induced by oxotremorine, arecoline, and carbamylcholine, respectively) (Figure 8). Furthermore, the fraction of carbamylcholine-stimulated [^{3}H]InsP accumulation recorded in the presence of oxotremorine was highly sensitive to TTX (Figure 8). This is again reminiscent of the findings in the cerebral cortex. Thus, although the interactions of agonists with the muscarinic receptor and of the agonist–receptor complex with a GTP-binding protein differ markedly between these two tissues (Gurwitz et al., 1985), it seems that the mechanism by which muscarinic agonists stimulate phosphoinositide hydrolysis may be essentially similar in the brain and the heart. However, some tissue-related differences in the coupling of muscarinic receptors to phosphoinositide hydrolysis (e.g., due to different G-proteins, muscarinic receptor subtypes, or differences in the membrane lipid environment) may exist. Such differences may account for the smaller fraction of oxotremorine/carbamylcholine-stimulated phosphoinositide hydrolysis in the cerebral cortex vs. the atrium (15% and 25%, respectively).

In conclusion, the studies presented here suggest that hydrolysis of phophoinositides in rat cerebral cortex and in heart atrium may proceed by two parallel and synergistic pathways, both of them mediated by muscarinic receptors. Different muscarinic agonists preferentially utilize one or another of these pathways, which might explain differences in their potency.

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Registry No. Ca, 7440-70-2; Na, 7440-23-5; carbamylcholine, 462-58-8; oxotremorine, 70-22-4; arecoline, 63-75-2; pilocarpine, 92-13-7.

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