Effect of Membrane Depolarization by High K^+ on Carbachol-Stimulated Phosphoinositides Hydrolysis in Guinea Pig Cerebral Cortical Slices

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Abstract—Stimulation of phosphoinositide hydrolysis by carbachol was studied in slices of guinea pig cerebral cortex under normal conditions (4.7 mM K^+) and depolarization conditions with high K^+ (42 mM K^+). Slices were labeled with [myo- 3H]-inositol, and the effects of carbachol and high K^+ on the formation of inositol-bisphosphates (IP_2) and inositol-trisphosphates (IP_3) were determined. Carbachol (10 mM) caused only 140% stimulation of the formations of IP_2 and IP_3 over the control value in normal Krebs Ringer Buffer (KRB), but about 200% stimulation in high K^+ medium. Dose-response curves for the effect of carbachol on the formations of IP_2 and IP_3 showed that high K^+ medium selectively decreased the ED50 value of carbachol for IP_2 formation about 3-fold. A Ca^{++} channel blocker, verapamil, inhibited the synergistic effect of carbachol and high K^+ on IP_2 formation, and a decrease in extracellular Ca^{++} also inhibited IP_2 formation induced by high K^+ , but these treatments had little, if any, effect on IP_3 formation. The possibility that IP_2 may be directly generated by hydrolysis of phosphatidylinositol 4-monophosphate (PIP) as well as from hydrolysis of IP_3 was discussed.

The responses to various hormone- and neurotransmitter-induced activations of phospholipase C and subsequent hydrolysis of membrane-bound phosphatidylinositol-4,5-bisphosphate result in the production of the second messengers inositol-1,4,5-trisphosphate and diacylglycerol (1, 2). The products of this reaction, inositol-1,4,5-trisphosphate and diacylglycerol, in turn release Ca^{++} from intracellular stores and activate protein kinase C, respectively (3, 4). In nerve tissue, not only neurotransmitters such as acetylcholine, but also depolarization is reported to lead to an increase in the metabolism of phosphoinositides (PI). For example, high K^+ or electrical stimulation increases PI hydrolysis (5, 6). Under certain conditions, depolarizing stimuli can induce PI hydrolysis by releasing endogenous acetylcholine (7) or by causing entry of calcium through dihydropyridine-

sensitive channels (8).

In the present study, using guinea pig cerebral cortical slices, we examined the effect of the muscarinic agonist carbachol on PI hydrolysis in normal and high K^+ medium and compared the stimulation of PI hydrolysis induced by receptor activation with that induced by depolarization.

Materials and Methods

Chemicals: [1,2- 3H]-Myoinositol (57.9–61.2 Ci/mmol) was purchased from New England Nuclear. Carbachol, atropine and verapamil were from Sigma Chemical Co.

Preparation of slices: Male guinea pigs, weighing 250 to 350 g, were sacrificed by decapitation. The brain was quickly removed and 350×350- μm slices of the cerebral cortex were cut in two perpendicular directions with a McIlwain tissue chopper at 4°C. The slices were suspended in 20 ml of Krebs-Ringer bicarbonate buffer solution (KRB)

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containing 124 mM NaCl, 3.45 mM KCl, 1.5 mM CaCl_2 , 1.25 mM KH_2PO_4 , 11 mM D-glucose and 25 mM NaHCO_3 , bubbled with 95% O_2 -5% CO_2 . For removal of damaged cells and subcellular debris, the slices were washed four or five times with KRB by allowing them to settle and then replacing the supernatant with fresh KRB. All procedures were done on ice.

Assay of inositol phosphate formation in cortical slices: Cortical slices (prepared from 1.2 g of cerebral cortex) were preincubated in 30 ml of fresh KRB for 30 min at 37°C. Then they were washed and incubated with labeled inositol (6 $\mu\text{Ci/ml}$) for 3 hours at 37°C. All the procedures were performed under continuous bubbling with 95% O_2 -5% CO_2 .

The slices were then washed six times with 20 ml of fresh KRB in which a part of the NaCl was replaced by 10 mM LiCl. Aliquots of 400 μl of the slice suspension (40–60 mg wet weight of tissue) were transferred to 12 $\times$ 75-mm glass tubes. Unless otherwise indicated, the slices were prewarmed for 5 min in 1 ml of KRB at 37°C and then incubated for 5 min in the presence of various agents. In medium in which the K^+ concentration was raised, the Na^+ concentration was decreased by an equivalent amount to maintain isotonicity. In some cases, Ca^{++} -free KRB containing 10 mM LiCl was used for the washes and resuspension, and Ca^{++} -EGTA buffer was added to the suspension of slices to obtain the indicated concentration of free Ca^{++} . The reaction was stopped after 5 min by adding 110 μl of 60% trichloroacetic acid (TCA) to give a final concentration of 6% TCA, and then the tubes were placed in ice-cold water. The slices were then homogenized in a Polytron. The homogenate was centrifuged at 500 $\times$ g for 20 min to obtain the supernatant. The pellet was then washed with 500 μl of 6% TCA and then centrifuged to obtain the supernatant. The two supernatants were combined and washed five times with 4 volumes of ethyl ether to remove TCA. Then they were supplemented with 4 ml of H_2O and applied to a column of 500 μl of Dowex AG 1-X8 (200–400 mesh, formate form). Inositol phosphates were eluted as described by Dones et al. (9). Briefly, IP_1 , IP_2 , IP_3 and IP_4 were eluted with

2 $\times$ 3 ml of 0.1 M formic acid containing 0.2, 0.4, 0.8 and 1.2 M ammonium formate, respectively. The radioactivity in the TCA precipitate was determined by the method of Gusovsky et al. (10) as a measure of [^3H]-inositol incorporation into phosphoinositides. The results are expressed as % ratios of [^3H]-inositol phosphates per total radioactivity incorporated into the TCA precipitate.

Data analysis: ED50 values were determined by computed non-linear least square regression (11). All values are means $\pm$ S.E.'s for the indicated numbers of experiments done in duplicate or triplicate. Significance was evaluated by Student's *t*-test.

Results

Time course of inositol phosphate formation stimulated by carbachol under normal and high K^+ conditions: Figure 1 shows the time courses of generation of IP_2 and IP_3 by 10 mM carbachol in normal and high K^+ (42 mM) KRB. Carbachol caused about a 140% increase in the formation of IP_2 and IP_3 within 5 min, and its stimulatory effects persisted for at least 10 min (Fig. 1, A and B). IP_1 formation did not change significantly during the incubation (data not shown). In high K^+ medium, the increases in IP_2 and IP_3 generations induced by carbachol were much greater (Fig. 1, C and D), and formation of IP_1 showed a similar change to that of IP_2 (data not shown). Atropine at 10 μM abolished the effect of carbachol on inositol phosphate formation under normal and high K^+ conditions, but had no effect on the high K^+ -induced generation of IP_2 and IP_3 (data not shown).

Dose-dependence of the carbachol on inositol phosphate formation: The dose-response curves for the effect of carbachol on the formation of IP_2 and IP_3 in normal and high K^+ medium are shown in Fig. 2. The concentrations of carbachol for half-maximal formations of IP_2 and IP_3 in normal medium were both 180 μM . In high K^+ medium, the ED50 of carbachol for IP_2 formation was decreased from 210 $\pm$ 54 μM to 66 $\pm$ 13 μM (mean $\pm$ S.E., $n=3$). This result shows that high K^+ raised the sensitivity to carbachol together with an increase in maximal response in IP_2 formation.

Fig. 1. Time course of PI hydrolysis stimulated by carbachol in normal KRB and in high K^+ medium. Slices were prewarmed at 37°C for 5 min, and then 10 mM carbachol (CCh) was added and incubation was carried out for the indicated times in the normal KRB (A and B) or in the high K^+ medium (C and D). Data are representative of three experiments. Values are means $\pm$ S.D.'s of triplicate measurements.

Effect of verapamil on carbachol- and high K^+ -stimulated inositol phosphate formation:

As shown in Fig. 3A, after a 5-min stimulation by 10 mM carbachol in KRB containing 1.5 mM Ca^{++} , IP_2 formation was about 140% of the control value and after a 5-min stimulation by high K^+ , IP_2 formation was about 410% of the control value. Incubation with carbachol and high K^+ together for 5 min resulted in synergistic enhancement of IP_2 formation to about 880% of the control value. In the presence of 100 μM verapamil, a Ca^{++} channel antagonist, carbachol-stimulated IP_2 formation was not changed, but the stimulation of IP_2 formation by high K^+ and by high K^+ plus carbachol were both inhibited about 50%. On the contrary, verapamil did not have any significant effect on IP_3 formation stimulated by high K^+ or by high K^+ plus carbachol (Fig. 3B).

Tetrodotoxin (10 μM) had no effect on phenomena.

Comparison of the effects of carbachol and high K^+ on inositol phosphate formation at different Ca^{++} concentrations: Slices were incubated with 10 mM carbachol or in high

K^+ medium containing 1.5 mM or 0.5 μM Ca^{++} for 5 min. As shown in Fig. 4, in the presence of 1.5 mM Ca^{++} , high K^+ stimulated the formation of IP_2 and IP_3 formation to 400% and 180%, respectively, of the control levels, while carbachol stimulated formations of both to 140% of the control values. A decrease in the Ca^{++} concentration to 0.5 μM resulted in about 60% inhibition of high K^+ -stimulated IP_2 formation with no change in IP_3 formation. The carbachol-stimulated formation of IP_2 and IP_3 were similar in 0.5 μM Ca^{++} medium to those in 1.5 mM Ca^{++} medium.

Discussion

In this study, we investigated the synergistic effects of carbachol and high K^+ on PI hydrolysis (mainly IP_2 and IP_3 formations) in guinea pig cerebral cortical slices. IP_4 formation was also monitored, but the incorporated radioactivity was very low and did not show any marked change (data not shown). The present experiments demonstrated that in normal medium, muscarinic stimulation by carbachol for 5 min enhanced the formations

Fig. 2. Dose-dependences of stimulations of formations of IP_2 and IP_3 by carbachol in normal KRB and high K^+ medium. Slices were prewarmed at $37^\circ C$ for 5 min, and then carbachol (CCh) was added at the indicated concentrations and incubation was carried out for 5 min in normal KRB (○) or high K^+ medium (●). Values are means $\pm$ S.E.'s for three experiments which were performed in triplicate. Hundred percent of each curve was taken from the response by 10^{-2} M carbachol under each condition; the actual value of the response to 10^{-2} M was similar to that shown in Fig. 1.

of IP_2 and IP_3 only slightly, but that in high K^+ medium, its stimulation of those formations was dramatically enhanced. These findings are consistent with those reported by Baird and Nahorski (12) and Eva and Costa (13). A receptor-mediated mechanism of activation of polyphosphoinositide-specific PLC through a guanine nucleotide binding protein is now becoming clear. Recent evidence indicates that in excitable tissues a rise in cytosolic Ca^{++} can also activate the PLC and that activation of PLC by Ca^{++} can be a direct effect, rather than a result of Ca^{++} -dependent release of neurotransmitters which activate PLC through a receptor-mediated mechanism (14, 15).

On depolarization of nerve cells, extracellular Ca^{++} is known to enter the cells through

Fig. 3. Effects of verapamil on formations of IP_2 and IP_3 stimulated by carbachol and high K^+ . Slices with (hatched columns) or without (open columns) pre-treatment with $100 \mu M$ verapamil at $37^\circ C$ for 5 min were incubated for 5 min in the presence of carbachol, high K^+ or both. Values are means $\pm$ S.E.'s for 4 experiments, which were done in duplicate or triplicate. ** $P < 0.01$; *** $P < 0.001$, significantly different from the corresponding value without verapamil.

voltage-dependent Ca^{++} channels. Moreover, phosphoinositide breakdown increases on elevation of the intracellular Ca^{++} level (16, 17). We found that the synergistic effect of carbachol and high K^+ medium on IP_2 formation was greater than that on IP_3 formation. Furthermore, the Ca^{++} channel blocker verapamil inhibited synergistic enhancement of IP_2 formation more effectively than that of IP_3 formation. Our data also showed that the ED_{50} of carbachol for stimulation of IP_2 formation by carbachol in high K^+ medium was one-third of that in normal KRB, but that its the ED_{50} for IP_3 formation in high K^+ medium was similar to that in normal KRB. These findings suggest that the increase in cytosolic Ca^{++} induced by high K^+ may increase the sensitivity of PLC to receptor activation, especially for IP_2 formation. The

Fig. 4. Effect of calcium deprivation on carbachol- and high K⁺-stimulated formation of IP₂ and IP₃. Slices were preincubated for 5 min in KRB containing Ca²⁺ at 1.5 mM (left part) or 0.5 μM (right part) and then incubated for 5 min with carbachol (10 mM, open columns) or high K⁺ (42 mM, hatched columns). Control values in KRB containing 1.5 mM Ca²⁺ were 2.7±0.2 (IP₂) and 1.5±0.1 (IP₃), and in KRB containing 0.5 μM Ca²⁺, they were 2.6±0.2 (IP₂) and 1.6±0.2 (IP₃) (dpm/10² dpm in lipids). Values are means±S.E.'s for 4 experiments carried out in duplicate or triplicate. ***P<0.001, significantly different from the corresponding value in 1.5 mM Ca²⁺.

effect of this cytosolic Ca²⁺ is probably distal to the muscarinic receptor, as high K⁺ does not alter the binding characteristics of the receptor (data not shown).

The following findings strongly suggest that most of the IP₂ formation stimulated in high K⁺ medium with or without carbachol was due to direct hydrolysis of PIP by activation of PLC and that dephosphorylation of IP₃ plays a minor role in this phenomenon: 1) When carbachol was added in high K⁺ medium, the formation of IP₂ was far greater than the formation of IP₃ which lasted continuously from 15 sec to 10 min (Fig. 1). 2) The stimulation of IP₃ formation by carbachol plus high K⁺ was inhibited more than that of IP₃ formation by verapamil, indicating that IP₂ formation was, to some extent, regulated independently of IP₃ formation. 3) High K⁺-stimulated formation of IP₂ was much higher than that of IP₃ and was more sensitive to a change in extracellular Ca²⁺ concentration (Fig. 4).

From our experiment, we cannot deny the presence of a large peak of IP₃ formation within 15 sec, but it is difficult to attribute the continuous increase of IP₂ formation for 10 min to this IP₃ peak.

Rhee's group reported the purifications of three types of PLC (PLC-I, PLC-II and PLC-III) from bovine brain (18, 19). Homma et al. reported that PLC-II and PLC-III have very different biochemical properties, such as different dependency on Ca²⁺ and substrate specificity. At low Ca²⁺ concentration (micromolar order), PLC-II and PLC-III hydrolyze only PIP₂, releasing IP₃ and diacylglycerol, but at higher Ca²⁺ concentration (millimolar concentration), PLC-II directly hydrolyzes PIP and PI, releasing IP₂ and IP₁ and diacylglycerol (20).

Even a high dose of verapamil could not completely inhibit IP's formation (Fig. 3). Sasakawa et al. reported that nifedipine inhibited high K⁺-induced IP₃ accumulation completely in cultured adrenal chromaffin cells (21). This discrepancy may be explained by the larger population of the Ca channels insensitive to the Ca channel antagonist in the brain.

We also found that the synergistic stimulatory effect on IP₃ formation by carbachol and high K⁺ was not inhibited effectively by verapamil, but was inhibited by the addition of EGTA to chelate all the extracellular Ca²⁺ (data not shown). These findings indicated that the synergistic effect on IP₂ and IP₃ formations has different sensitivity to Ca channel antagonists.

Activation of PLC by Ca²⁺, resulting in direct hydrolysis of PIP, may be a physiological mechanism for diacylglycerol generation and protein kinase C activation following Ca²⁺ influx. Thus, the regulation of PLC activity by Ca²⁺ may be physiologically important in regulating the activity of protein kinase C in excitable tissues.

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