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Activation of phospholipase C in SH-SY5Y neuroblastoma cells by potassium-induced calcium entry

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1 We used SH-SY5Y human neuroblastoma cells to investigate whether depolarization with high K^+ could stimulate inositol (1,4,5)trisphosphate ($Ins(1,4,5)P_3$) formation and, if so, the mechanism involved.

2 $Ins(1,4,5)P_3$ was measured by a specific radioreceptor mass assay, whilst $[Ca^{2+}]_i$ was measured fluorimetrically with the Ca^{2+} indicator dye, Fura-2.

3 Depolarization with K^+ caused a time- and dose-dependent increase in $[Ca^{2+}]_i$ (peak at 27 s, EC_{50} of 50.0 ± 9.0 mM) and $Ins(1,4,5)P_3$ formation (peak at 30 s, EC_{50} of 47.4 ± 1.1 mM).

4 Both the K^+-induced $Ins(1,4,5)P_3$ formation and increase in $[Ca^{2+}]_i$ were inhibited dose-dependently by the L-type voltage-sensitive Ca^{2+} channel closer, (R+)-BayK8644, with IC_{50} values of 53.4 nM and 87.9 nM respectively.

5 These data show a close temporal and dose-response relationship between Ca^{2+} entry via L-type voltage-sensitive Ca^{2+} channels and $Ins(1,4,5)P_3$ formation following depolarization with K^+, indicating that Ca^{2+} influx can activate phospholipase C in SH-SY5Y cells.

Keywords: Inositol(1,4,5)trisphosphate; phospholipase C; depolarization; SH-SY5Y human neuroblastoma cells; potassium; L-type voltage-sensitive Ca^{2+} channels

Introduction

It is now well established that many G-protein coupled receptors activate phospholipase C (PLC), and that the products of polyphosphoinositide hydrolysis are important intracellular messengers, especially inositol(1,4,5)trisphosphate ($Ins(1,4,5)P_3$), which releases Ca^{2+} from internal stores (for review see Berridge, 1993). Furthermore, it has been shown that depolarization induced by elevated extracellular K^+ or veratrine can also stimulate PLC in both neuronal (Kendall & Nahorski, 1984; 1985; 1987; Baird & Nahorski 1986; 1990; Chandler & Crews, 1990) and non-neuronal (Biden *et al.*, 1987; Eberhard & Holz 1987; 1991; Kelley *et al.*, 1994) preparations. However, the neuronal tissues used in these studies were heterogeneous (i.e. cerebral-cortex slices or synaptosomes) in their cellular composition and depolarization might have indirectly stimulated phosphoinositide hydrolysis by releasing endogenous neurotransmitters capable of activating PLC. Indeed, Baird & Nahorski (1990) concluded that a significant proportion of the K^+-depolarization induced increase in inositol polyphosphates seen in cerebral-cortex slices was due to such an indirect activation of PLC.

However, in addition to this indirect, receptor-mediated mechanism, there is also evidence for the activation of PLC by K^+-induced Ca^{2+} influx (Chandler & Crews, 1990). Several groups have shown that Ca^{2+} entry via L-type voltage-sensitive calcium channels (VSCCs) may be involved in either of these mechanisms of PLC activation (Kendall & Nahorski, 1985; Gonzales *et al.*, 1989; Hajnoczky *et al.*, 1992).

The homogeneous neuroblastoma cell-line, SH-SY5Y, possesses both L- and N-type VSCCs (Morton *et al.*, 1992), as well as receptor operated Ca^{2+} channels (Lambert & Nahorski, 1990). Furthermore, we have previously demonstrated in SH-SY5Y cells that the plateau phase of carbachol-induced $Ins(1,4,5)P_3$ formation is extracellular Ca^{2+} dependent (Lambert *et al.*, 1991a), indicating that these cells possess a Ca^{2+}-sensitive isoform of PLC. The present study was designed to investigate whether K^+-induced Ca^{2+} entry was sufficient to stimulate $Ins(1,4,5)P_3$ formation, and to determine the type(s) of Ca^{2+} channel involved. We have shown that depolarization with high K^+ opens L-type VSCCs, allowing Ca^{2+} influx to activate PLC.

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Methods

Cell culture and harvesting

Undifferentiated SH-SY5Y human neuroblastoma cells (passage 65–80) were cultured in minimum essential medium with Earle's salts supplemented with 2 mM L-glutamine, 100 uml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, 2.5 $\mu g\ ml^{-1}$ fungizone, and 10% foetal calf serum.

Cells were harvested with 10 mM HEPES-buffered saline/0.02% EDTA, pH 7.4, washed twice with, and then resuspended, in Krebs/HEPES buffer, pH 7.4, of the following composition (in mM), unless stated otherwise below: Na^+ 143.3, K^+ 4.7, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 125.6, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, glucose 11.7 and HEPES 10. For K^+ stimulation experiments the $[Na^+]$ of the buffer was adjusted accordingly, to maintain tonicity.

Measurement of Ins(1,4,5)P_3

Whole cell suspensions (final volume 0.3 ml) were pre-incubated at 37°C, with or without the L-type VSCC blocker (R+)-BayK8644 (1 nM–10 μM; Van Amsterdam *et al.*, 1989) for 15 min. The cells were then incubated in the presence of K^+ (0–100 nM added) or ionomycin (1 μM) for 0–300 s. Reactions were terminated by the addition of 0.3 ml of 1 M trichloroacetic acid. $Ins(1,4,5)P_3$ was extracted with Freon/octylamine (1:1, vol/vol) and neutralized with 25 mM $NaHCO_3$. $Ins(1,4,5)P_3$ was assayed using a bovine adrenocortical binding protein and $[^3H]$-$Ins(1,4,5)P_3$ (41 $Cimmol^{-1}$) at 4°C. Authentic $Ins(1,4,5)P_3$ (0.036–12 pmol) in buffer, taken through an identical extraction procedure, was used as a standard. Nonspecific binding was defined in the presence of excess $Ins(1,4,5)P_3$ (0.3 nmol). Bound $[^3H]$-$Ins(1,4,5)P_3$ was separated by rapid vacuum filtration (Challiss *et al.*, 1988).

Measurement of $[Ca^{2+}]_i$

This was done fluorimetrically with the Ca^{2+} indicator dye Fura-2, as described previously (Lambert & Nahorski, 1990). Briefly, cells were incubated at 37°C with 3 μM Fura-2/AM for 30 min, then washed and postincubated at 20°C for 20 min, to allow complete ester hydrolysis. Some cells were preincubated with (R+)-BayK8644 (1 nM–10 μM) at 20°C for 15 min immediately prior to use. $[Ca^{2+}]_i$ was measured in 2 ml suspensions of Fura-2 loaded cells at 37°C in a Perkin-Elmer LS50B spectrofluorimeter, using 340/380 nm excitation with emission at 510 nm. $[Ca^{2+}]_i$ was then calculated from the 340/380 ratio according to Grynkiewicz *et al.* (1985), where R_{max} and R_{min} were determined with Triton-X (0.1%) and EGTA (4.5 mM, pH > 8.0) respectively.

Sources of reagents

All cell culture materials were supplied by Gibco, U.K. (R+)-BayK8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) was obtained from RBI, U.S.A. via SEMAT technical supplies, U.K. [^{3}H]-Ins(1,4,5)P_3 was supplied by Amersham, U.K. Fura-2/AM and all other reagents were obtained from Sigma, U.K.

Data analysis

All data are given as mean ± s.e.mean unless otherwise stated. EC_{50} (half maximal stimulation) and IC_{50} (half maximal inhibition) values were obtained by computer-assisted curve (non-linear regression model) fitting using GRAPHPAD, and where given as mean ± s.e.mean are calculated from replicate analyses. Statistical comparisons were made where appropriate by Student's *t* test and/or ANOVA and were considered to be significant when $P < 0.05$.

Results

Depolarization with K^+ (50 mM added) caused a monophasic increase in Ins(1,4,5)P_3 formation, which peaked (14.3 ± 0.5 pmol mg^{-1} protein, $n = 5$) at 30 s and returned to basal levels (8.1 ± 1.0 pmol mg^{-1} protein, $n = 5$) between 1 and 2 min (Figure 1). This stimulation of Ins(1,4,5)P_3 formation by K^+ (measured at the peak) was dose-dependent (Figure 1, inset), with an EC_{50} of 47.4 ± 1.1 mM. Depolarization with K^+ (50 mM added) also caused an increase in $[Ca^{2+}]_i$ (from 112.0 nM), which peaked (166.8 nM) at 27 s (Figure 2). This K^+-induced increase in $[Ca^{2+}]_i$ (measured at the peak) was also dose-dependent (Figure 2, inset), with an EC_{50} of 50.0 ± 9.0 mM ($n = 5$).

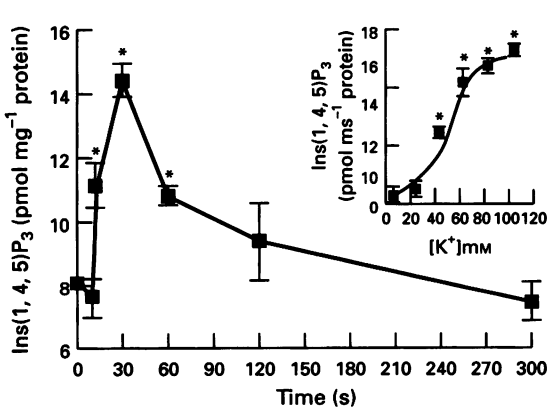

Figure 1 Potassium-induced Ins(1,4,5)P_3 formation in SH-SY5Y cells. Main panel depicts time course of Ins(1,4,5)P_3 formation following depolarization with K^+ (50 mM added). Inset shows the dose-dependency of this response at the peak (30 s). Whole cell suspensions (final volume 0.3 ml) were preincubated at 37°C for 15 min, and then incubated in the presence of K^+ (0–100 mM added) for 0–300 s. Ins(1,4,5)P_3 was measured by a specific radioreceptor mass assay. Data are mean ± s.e.mean where $n = 5$. *denotes $P < 0.05$ (*t* test) increase compared to basal.

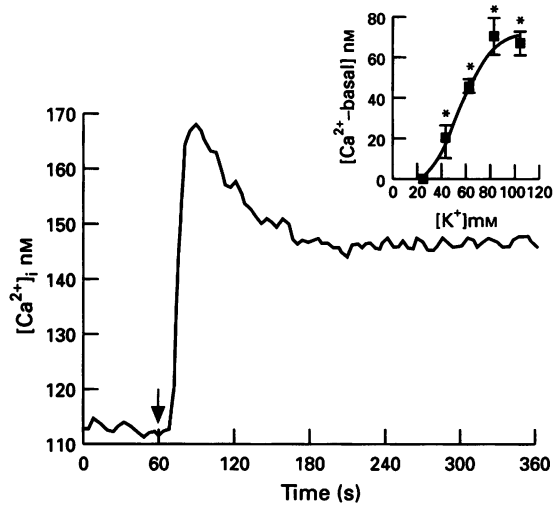

Figure 2 Potassium-induced increase in $[Ca^{2+}]_i$ in SH-SY5Y cells. Main panel is a typical trace (of $n = 5$) depicting $[Ca^{2+}]_i$ before and after K^+ (50 mM added), given at time indicated by arrow. Inset shows the dose-dependency of this response at the peak (mean ± s.e.mean, $n = 5$). Cells were loaded with the Ca^{2+} indicator dye, Fura-2 and the $[Ca^{2+}]_i$ measured by fluorimetry before and after K^+ (0–100 mM added). *denotes $P < 0.05$ (*t* test) increase compared with basal.

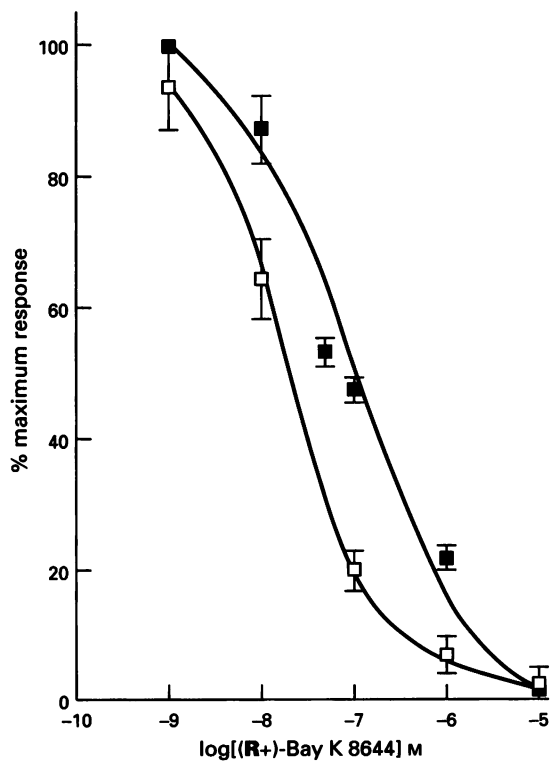

Figure 3 Inhibition by (R+)-Bay K 8644, an L-type VSCC blocker, of potassium-induced Ca^{2+} influx (■) and Ins(1,4,5)P_3 formation (□). Cells were preincubated with or without (R+)-Bay K 8644 (1 nM–10 μM) for 15 min, and then incubated with K^+ (50 mM added). Data are mean ± s.e.mean, where $n = 5$–13. Basal Ins(1,4,5)P_3 formation was 6.3 ± 0.4 pmol mg^{-1} protein. Maximum response (= 100%) was defined as the increase in $[Ca^{2+}]_i$/Ins(1,4,5)P_3 caused by K^+ (50 mM added) alone.

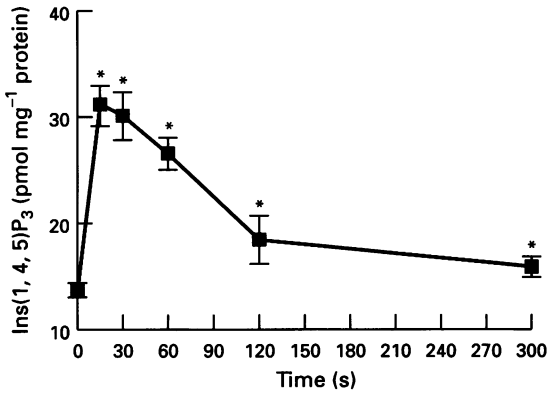

Figure 4 Ionomycin-induced Ins(1,4,5)P_3 formation in SH-SY5Y cells. Whole cell suspensions (final volume 0.3 ml) were preincubated at 37°C for 15 min, and then incubated with ionomycin (1 μM) for 0–300 s. Ins(1,4,5)P_3 was measured by a specific radioreceptor mass assay. Data are mean ± s.e.mean, where $n=5$. *denotes $P<0.05$ (*t* test) increase compared to basal.

Preincubation (15 min) with the L-type VSCC blocker (**R**+)-BayK8644 dose-dependently inhibited K^+ (50 mM added) depolarization-induced Ins(1,4,5)P_3 formation (Figure 3), with an IC_{50} of 53.4 ± 4.3 nM ($n=5$). Preincubation with (**R**+)-BayK8644 also caused a dose-dependent inhibition of the K^+ (50 mM added)-induced increase in $[Ca^{2+}]_i$ (Figure 3), with an IC_{50} of 87.9 nM (obtained from a composite curve of $n=5$–13 points), indicating that Ca^{2+} entry occurs via L-type VSCCs.

Ionomycin (1 μM), a Ca^{2+} ionophore, stimulated Ins(1,4,5)P_3 formation, which peaked (31.0 ± 1.9 pmol mg^{-1} protein, $n=5$) at 15 s and then declined to 15.7 ± 0.9 pmol mg^{-1} protein ($n=5$) from 2–5 min (Figure 4). Ionomycin (1 μM) also caused a rapid increase in $[Ca^{2+}]_i$ (approaching R_{max} and estimated at 5 μM), which was sustained until 300 s, when sampling ended (data not shown).

Discussion

We show here that depolarization with high extracellular K^+ opens L-type VSCCs, allowing Ca^{2+} influx to activate PLC, resulting in increased Ins(1,4,5)P_3 formation, in a homogeneous neuronal preparation. Although SH-SY5Y cells release noradrenaline when stimulated with high K^+ (Atcheson *et al.*, 1994), these cells do not possess PLC-coupled α_1-adrenoceptors, only α_2-receptors, which do not couple to polyphosphate turnover (Smart *et al.*, 1995). Therefore, the activation of PLC by K^+ must occur via a non-receptor-mediated mechanism. Furthermore, ionomycin, a Ca^{2+} ionophore, also stimulated Ins(1,4,5)P_3 formation. Earlier studies have also suggested a role for the non-receptor-mediated activation of PLC by K^+-induced Ca^{2+} entry (Chandler & Crews, 1990). Indeed, Challiss & Nahorski (1991) showed that only a proportion of the K^+-induced increase in Ins(1,4,5)P_3 formation in rat brain slices was atropine-reversible, whilst concomitant blockade of VSCCs with nitrendipine further suppressed the response. In addition, it has been reported that K^+-induced Ca^{2+} influx stimulates PLC in primary cultures of rat adrenal glomerulosa cells (Hajnoczky *et al.*, 1992). It is also worthy of note that depolarization with K^+ may have increased the amount of PLC's substrate, phosphatidyinositol bisphosphate, as previously shown in adrenal chromaffin cells (Eberhard & Holz, 1991).

The current finding that K^+-induced depolarization stimulated Ins(1,4,5)P_3 formation in SH-SY5Y cells is of further interest as a previous study in SK-N-SH cells (the parent cell-line to SH-SY5Y) failed to detect such changes using the measurement of total [^{3}H]-inositol polyphosphate accumulation in the presence of Li^+ (Baird *et al.*, 1989). This apparent discrepancy is most likely due to the fact that the K^+-induced increase in Ins(1,4,5)P_3 levels is both small and brief, so would contribute very little to total polyphosphate turnover and thus be masked by basal accumulation. Indeed, we have previously found that the Li^+ block technique failed to detect similar small, transient changes in Ins(1,4,5)P_3 formation caused by opioids or halothane in SH-SY5Y cells (Smart *et al.*, 1994a,b). Furthermore, Zhang & Melvin (1993) demonstrated that, in rat salivary acinar cells, depolarization with K^+ stimulated total polyphosphoinositide hydrolysis without affecting Ins(1,4,5)P_3 levels, presumably by simultaneously enhancing its conversion by 3-kinase to Ins(1,3,4,5)P_4. Whilst 3-kinase is Ca^{2+}-sensitive, its sensitivity is lower than that of PLC (see Shears, 1989). In SH-SY5Y cells, PLC was activated at 12 fold lower concentrations of Ca^{2+} than 3-kinase (Lambert *et al.*, 1991b). However, K^+-induced stimulation of 3-kinase activity has been reported for rat cerebral-cortex preparations (Challiss & Nahorski, 1991).

Depolarization with K^+ dose-dependently stimulated Ins(1,4,5)P_3 formation, with an EC_{50} of 47 mM. This dose-response to K^+, whilst appearing weak, is consistent with our previous studies of K^+-stimulated noradrenaline release (63 mM) and adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation (49 mM) (Atcheson *et al.*, 1994), as well as the K^+-induced increase in $[Ca^{2+}]_i$ (50 mM) in the present study, indicating the importance of Ca^{2+} in cross-talk between second messenger systems.

K^+-depolarization also increased $[Ca^{2+}]_i$ in a dose-dependent manner, by opening VSCCs and so allowing Ca^{2+} influx. There was a close temporal and dose relationship between K^+-induced Ca^{2+} entry and the increase in Ins(1,4,5)P_3 formation, suggesting that Ca^{2+} influx activates PLC. There are several Ca^{2+}-sensitive isoforms of PLC (Cockcroft & Thomas, 1992), and SH-SY5Y cells appear to express at least one, as the plateau phase of carbachol-induced Ins(1,4,5)P_3 formation is abolished by the removal of extracellular Ca^{2+} (Lambert *et al.*, 1991a). In addition, elevation of $[Ca^{2+}]_i$ with the Ca^{2+} ionophore, ionomycin, also stimulated Ins(1,4,5)P_3 formation. However, the relatively transient nature of these increases in Ins(1,4,5)P_3 formation in the presence of sustained increases in $[Ca^{2+}]_i$ suggests that the Ins(1,4,5)P_3 response rapidly desensitizes, probably at the level of the effector enzyme, PLC. Moreover, it is possible that Ins(1,4,5)P_3-induced Ca^{2+} mobilization from intracellular stores (Berridge, 1993) might also contribute to the increase in $[Ca^{2+}]_i$ at later time points.

There are several types of VSCCs (denoted L,N,T and P), each of which have different electrophysical and pharmacological properties (for review, see Spedding & Paoletti, 1992). However, SH-SY5Y cells possess only the L- and N- types of VSCCs, as indicated by the fact that both dihydropyridines and ω-conotoxin partially antagonize, whilst a combination of the two antagonists abolishes, the calcium current (Reeve *et al.*, 1994), although it is very difficult to detect the two separate currents by direct measurement of their electrical properties (Toselli *et al.*, 1991; Morton *et al.*, 1992; Reeve *et al.*, 1994). (**R**+)-BayK8644 dose-dependently inhibited K^+-induced Ins(1,4,5)P_3 formation, with an IC_{50} of 53 nM. At 1 μM (**R**+)-BayK8644 a 93% inhibition of the Ins(1,4,5)P_3 response was observed, indicating that the response was mediated by Ca^{2+} entry via L-type VSCCs (Van Amsterdam *et al.*, 1989). However, 1 μM (**R**+)-BayK8644 only inhibited Ca^{2+} influx by 80%, indicating N-type VSCCs were also activated by K^+, but did not admit sufficient Ca^{2+} to activate PLC. We have previously shown that K^+ opens both L and N-type VSCCs in SH-SY5Y cells and that the contribution of the N-type channel is small, although this increases with higher doses of K^+ (Lambert *et al.*, 1990). Ca^{2+} entry via L-type VSCCs has been implicated in K^+-induced polyphosphoinositide hydrolysis in rat cerebral-cortex preparations (Kendall & Nahorski, 1985; Gonzales *et al.*, 1989; Chandler & Crews, 1990), and it has been reported that in rat adrenal glomerulosa cells depolarization with K^+ activates PLC via Ca^{2+} entry through L-type

VSCCs (Hajnoczky *et al.*, 1992). It is also worth noting here that others have reported that different modes of Ca^{2+} influx (via L-type VSCCs or via NMDA-receptor operated channels) generate different intracellular signals in hippocampal neurones (Bading *et al.*, 1993).

In conclusion, this study demonstrates that K^+-induced depolarization causes a non-receptor-mediated stimulation of $Ins(1,4,5)P_3$ formation, secondary to the opening of L-type VSCCs and Ca^{2+}-induced activation of PLC, in SH-SY5Y human neuroblastoma cells.

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