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SHORT COMMUNICATION

SK&F 96365 (1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenylethyl}-1*H*-imidazole hydrochloride) Stimulates Phosphoinositide Hydrolysis in Human U373 MG Astrocytoma Cells

*J.-A. Arias-Montaño, W. J. Gibson and J. M. Young**

Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, U.K.

ABSTRACT. SK&F 96365 (1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenylethyl}-1*H*-imidazole hydrochloride) stimulated the accumulation of [^{3}H]inositol monophosphates ([^{3}H]IP_1) in human U373 MG astrocytoma cells prelabelled with [^{3}H]inositol (EC_{50} 15 ± 1 μM, Hill coefficient 3.8 ± 0.4). SK&F 96365-induced accumulation of [^{3}H]IP_1 increased linearly with time, but there was no initial rapid formation of [^{3}H]IP_3. SK&F 96365 also stimulated [^{3}H]IP_1 accumulation in human HeLa cells, but only to a small extent in slices of rat cerebral cortex and guinea-pig cerebellum. SK&F 96365-induced accumulation of [^{3}H]IP_1 in U373 MG cells increased as extracellular Ca^{2+} was increased from nominally zero to 4 mM, but there was no evidence that SK&F 96365 induced any marked entry of Ca^{2+} into cells; only an inhibition of store-refilling-induced Ca^{2+} entry was apparent. Further, the response to SK&F 96365 was additive with that to the Ca^{2+} ionophore ionomycin. Depolarization of the cells with raised K^+ produced only a small stimulation of phosphoinositide hydrolysis. SK&F 96365 caused the release of Ca^{2+} from intracellular stores in U373 MG cells (EC_{50} 26 ± 14 μM), but thapsigargin induced only a small accumulation of [^{3}H]IP_1. Miconazole, another *N*-substituted imidazole, also stimulated [^{3}H]IP_1 accumulation in U373 cells. BIOCHEM PHARMACOL **56**;8:1023–1027, 1998.

KEY WORDS. SK&F 96365; U373 MG astrocytoma cells; inositol phosphates; intracellular calcium; ionomycin; miconazole

SK&F 96365† was introduced as a blocker of receptor-operated Ca^{2+} channels [1], but it is known to block a variety of Ca^{2+} entry pathways, including Ca^{2+} entry through L-type voltage-dependent Ca^{2+} channels [1], I_{CRAC} channels activated by the emptying of intracellular Ca^{2+} stores [1–5], and the channels activated by maitotoxin [6]. Conversely, SK&F 96365 also induces release of Ca^{2+} from intracellular stores, via inhibition of the Ca^{2+}-ATPase [2, 7] and at higher concentrations can promote Ca^{2+} entry through non-selective cation channels, at least in human umbilical vein endothelial cells [8] and HL-60 cells [4]. In the endothelial cells SK&F 96365 also blocked an inwardly rectifying K^+ channel, thereby causing depolarization and a reduction in the driving force for Ca^{2+} entry [8]. In spite of these multiple actions SK&F 96365 is still widely used as a Ca^{2+} entry blocker, although the end-effect on Ca^{2+}-regulated processes in cells may not be easy to predict. This is illustrated for PLC-mediated hydrolysis of phosphoinositides, in which Ca^{2+} may have multiple roles [9, 10]. An increase in intracellular Ca^{2+} alone may be sufficient to cause inositol phosphate (IP) formation [11, 12] and SK&F 96365 has been shown to inhibit maitotoxin-induced IP formation in C6 glioma cells, presumably by blocking the maitotoxin-induced Ca^{2+} entry [6]. However, we report here that in human U373 MG astrocytoma cells SK&F 96365 itself stimulates phosphoinositide hydrolysis. Some of these results have been presented in preliminary form to the British Pharmacological Society [13].

MATERIALS AND METHODS

Measurement of [^{3}H]inositol Phosphates

The accumulation of [^{3}H]inositol phosphates in suspensions of U373 MG cells and HeLa cells and in cross-chopped slices of guinea-pig cerebellum and rat cerebral cortex, all prelabelled with [^{3}H]inositol, was measured as described previously [14]. For measurements on U373 MG cell monolayers, cells were seeded (approx. 30,000 cells/

* Corresponding author: Dr. J. M. Young, Department of Physiology, Biophysics and Neurosciences, Centro de Investigacion y de Estudios Avanzados del IPN, Apartado Postal 14–740, 07000 Mexico, D.F.; Tel. +44–1223-334035; FAX +44–1223-334040.

† *Abbreviations:* $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; [^{3}H]IP_1, [^{3}H]inositol monophosphates; [^{3}H]IP_2, [^{3}H]inositol bisphosphates; [^{3}H]IP_3, [^{3}H]inositol trisphosphates; [^{3}H]IP, total inositol phosphates; PLC, phospholipase C; and SK&F 96365, (1-{β-[3-(4-methoxyphenyl-)propoxy]-4- methoxyphenylethyl}-1H-imidazole hydrochloride).

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well) in 12-well plates and grown to near confluence. The culture medium was removed and the monolayers washed with 1 mL of inositol-free Dulbecco's Modified Eagle's Medium before addition of 0.5 mL of inositol-free Dulbecco's Modified Eagle's Medium containing 10% dialysed calf serum, 10 μM myo-inositol and 2.5 $\mu Ci \cdot mL^{-1}$ [^{3}H]inositol (0.16 μM). After 24 hr the medium was aspirated and the cells were washed once with Krebs–Henseleit buffer. Krebs–Henseleit buffer containing LiCl (30 mM) was added to each well and the cells incubated for 15 min at 37° before addition of agonist (final volume 0.5 mL). The incubation was terminated and labelled inositol phosphates separated as for cells in suspension.

Fluorometric Determination of Intracellular Ca^{2+} ($[Ca^{2+}]_i$)

Fluorometric measurements of $[Ca^{2+}]_i$ were made using an Hitachi F-2000 spectrometer as described elsewhere [15]. To reduce amounts of SK&F 96365 required, coverslips were superfused with 5 mL of the appropriate SK&F 96365-containing solution and the superfusion then stopped for a maximum of 10 min before superfusing again with HEPES medium alone. Parallel measurements in the absence of SK&F 96365 gave no indication that the responses of U373 MG cells were altered by this protocol.

Analysis of Data

Concentration-response data were fitted by non-linear regression to a Hill equation [14]. The statistical significance of differences between multiple treatments within the same experiment was assessed using either the Dunnett test or the Student–Newman–Keuls multiple range test. Where multiple values of mean ± SEM were obtained from independent experiments, the overall mean was calculated as the weighted mean ± SEM [16]. Where the SEM of a ratio is given, this is the approximate SEM obtained from the expression for the approximate variance of a function with more than one variable [16].

Chemicals

SK&F 96365 (1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenylethyl}-1*H*-imidazole HCl) was a kind gift from SmithKline Beecham Pharmaceuticals, Harlow, Essex CM19 5AW. CP-96345 (2s,3s-*cis*-3-(2-methoxybenzylamino)-2-benhydrylquinuclidine) was kindly provided by Dr K. Watling, Parke–Davis Neuroscience Research Centre.

RESULTS AND DISCUSSION

SK&F 96365-induced [^{3}H]inositol Phosphate Accumulation

SK&F 96365 (30 μM) induced the accumulation of [^{3}H]IP_1, [^{3}H]IP_2 and [^{3}H]IP_3 in [^{3}H]inositol labelled human U373 MG astrocytoma cells in the presence of 30 mM Li^+ (Fig. 1). The accumulation of [^{3}H]IP_1 was linear with time up to 60 min (Fig. 1A), the longest period studied, whereas for both [^{3}H]IP_2 and [^{3}H]IP_3 the rate of accumulation decreased with time (Fig. 1B and C). It is notable that SK&F 96365-induced [^{3}H]IP_3 accumulation gave no evidence of an initial rapid phase, in contrast to the time course observed for histamine-stimulated [^{3}H]IP_3 produc-

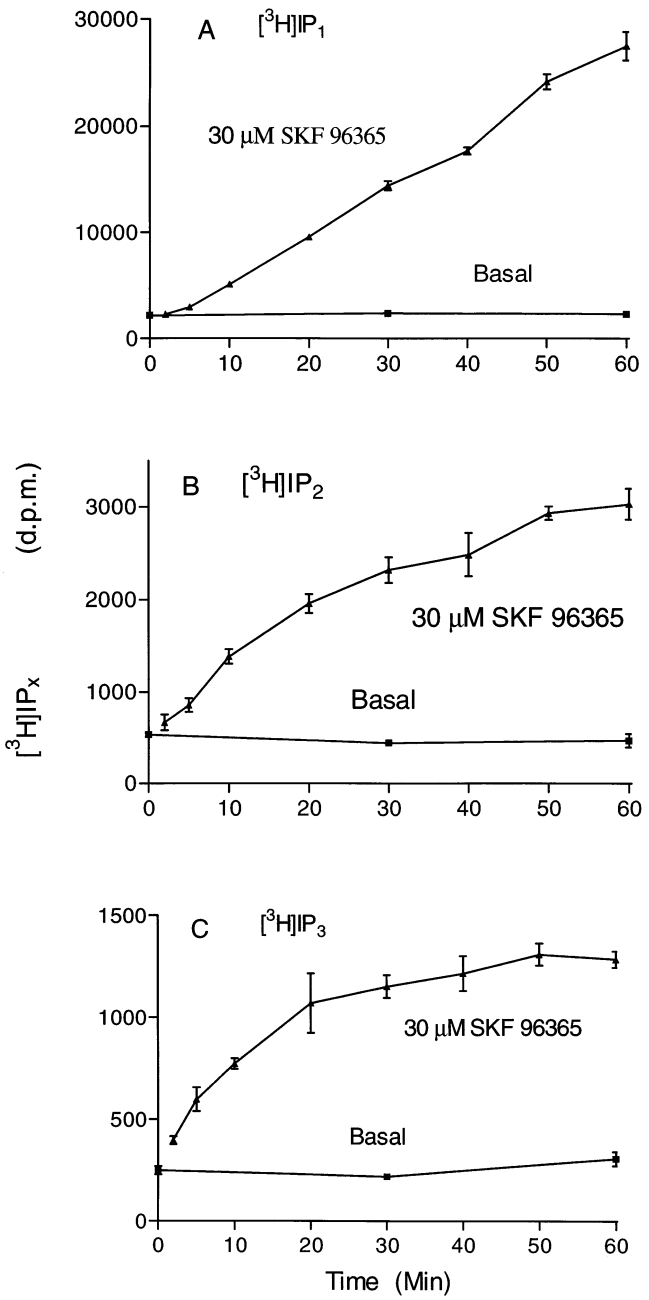

FIG. 1. Time course of SK&F 96365-induced [^{3}H]IP_x accumulation in U373 MG cells. Points are the means ± SEM of triplicate determinations within a single experiment in the presence or absence (basal) of 30 μM SK&F 96365. Where no error bars are shown the error was within the size of the symbol. The whole experiment was repeated twice more. (A) [^{3}H]IP_1 (B) [^{3}H]IP_2 (C) [^{3}H]IP_3.

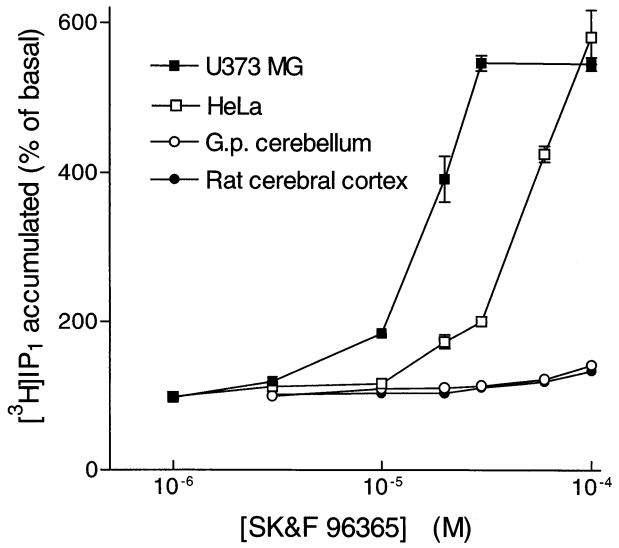

FIG. 2. SK&F 96365-induced [^{3}H]IP$_1$ accumulation in U373 MG cells, HeLa cells, rat cerebral cortical slices and guinea-pig cerebellar slices. The values are the combined data (weighted means ± SEM) from 3 independent experiments in each tissue. Where no error bars are shown the error was within the size of the symbol. Mean basal accumulations of [^{3}H]IP$_1$ (d.p.m.) were 1346 ± 56 (U373 MG), 4100 ± 341 (HeLa), 2290 ± 142 (rat) and 2943 ± 389 (guinea pig).

tion in these cells [14]. This makes it unlikely that SK&F 96365 is acting as an agonist at a G protein-coupled receptor. Consistent with this, [^{3}H]IP$_1$ accumulation in response to 30 μM SK&F 96365 was not significantly changed in the presence of mepyramine, methylatropine, prazosin or CP-96345, all at 1 μM, antagonists at receptors (histamine-H$_1$, muscarinic non-selective, α_1-adrenoceptor and NK$_1$-tachykinin, respectively) known to be coupled to phosphoinositide hydrolysis in these cells. After a 30-min incubation with 30 μM SK&F 96365, [^{3}H]IP$_1$ was much the major [^{3}H]IP fraction present and accounted for 83 ± 1% of total [^{3}H]IP$_1$ + [^{3}H]IP$_2$ + [^{3}H]IP$_3$ (N = 7). In most subsequent experiments with SK&F 96365 only the [^{3}H]IP$_1$ fraction was collected and incubations were routinely for 30 min.

The stimulation of [^{3}H]IP$_1$ accumulation by SK&F 96365 was concentration-dependent, with an EC_{50} of 15 ± 1 μM. The extent of the stimulation by 30–50 μM SK&F 96365 varied between 2.0- and 6.7-fold of basal over the course of the study. The concentration-response curve was much steeper (Hill coefficient 3.8 ± 0.4) than expected for a hyperbola, but this could reflect a second, inhibitory, action at high concentration. SK&F 96365 also stimulated [^{3}H]IP$_1$ accumulation in monolayers of U373 MG cells (EC_{50} 14 ± 1 μM, Hill coefficient 2.4 ± 0.2), indicating that the response in suspensions was not an artefact of dissociation.

SK&F 96365 also stimulated [^{3}H]IP$_1$ accumulation in human HeLa cells, but the concentration-response curve was to the right of that in U373 MG cells (Fig. 2), suggesting that the effect of SK&F 96365 is not due to a nonspecific action on cell function. However, the amount of [^{3}H]IP$_1$ accumulated in two brain tissues after 60-min incubation with 100 μM SK&F 96365 was relatively very small, 1.4 ± 0.1 and 1.3 ± 0.1 fold of basal (3) in guinea-pig cerebellar and rat cerebral cortical slices, respectively (Fig. 2), although statistically significant. Thus stimulation of phosphoinositide hydrolysis by SK&F 96365 can be detected in brain tissues, but a marked response is obtained only in the two transformed cell lines.

Ca^{2+}-dependence of SK&F 96365-stimulated [^{3}H]IP$_1$ Accumulation

[^{3}H]IP$_1$ accumulation stimulated by 30 μM SK&F 96365 increased as the extracellular Ca^{2+} concentration increased, even in the millimolar concentration range (Fig. 3A). A similar pattern was observed for [^{3}H]IP$_2$ (Fig. 3B) and [^{3}H]IP$_3$ (Fig. 3C). A facilitation of phosphoinositide hydrolysis by extracellular Ca^{2+} in the range 0 to *circa* 1.3 mM of Ca^{2+} appears to be common to all receptors coupled to PLC-β when stimulation by agonist is extended beyond 30–60 sec [9, 10], but the further statistically significant increase in [^{3}H]IP$_1$ stimulated by 30 μM SK&F 96365 between 1.3 and 4 mM Ca^{2+} (Fig. 3A) suggests that the response to SK&F 96365 is linked to Ca^{2+} in a more complex fashion.

Increases in $[Ca^{2+}]_i$ are known to stimulate phosphoinositide hydrolysis [11, 12] and SK&F 96365 has been reported to stimulate Ca^{2+} entry in human HL-60 cells, $EC_{50} > 30$ μM, [4] and in primary human endothelial cell cultures, EC_{50} 141 μM, [8], by a pathway distinct from that associated with the refilling of intracellular stores. However, when monolayers of U373 MG cells grown on coverslips were treated with 5 μM thapsigargin in nominally Ca^{2+}-free medium (no added Ca^{2+}) to empty intracellular Ca^{2+} stores, followed by superfusion with Ca^{2+}-containing medium, subsequent addition of 20–70 μM SK&F 96365 caused a rapid decline in $[Ca^{2+}]_i$ (as monitored by fura-2 fluorescence) to basal levels, consistent with the reported potency of SK&F 96365 as an inhibitor of capacitative Ca^{2+} entry, IC_{50} 3–28 μM [1–5]. There was no indication of induced Ca^{2+} entry at concentrations at which SK&F 96365 stimulates [^{3}H]IP$_1$ accumulation.

The conclusion that the effect of SK&F 96365 is not secondary to induced Ca^{2+} entry and direct stimulation of a PLC was strengthened by comparing the response to SK&F 96365 with that to the Ca^{2+} ionophore ionomycin, which stimulated [^{3}H]IP$_1$ accumulation in U373 MG cells with an EC_{50} of 1.4 ± 0.2 μM and a best-fit maximum response of 390 ± 16% of basal. However, the time course of [^{3}H]IP$_3$ accumulation induced by 3 μM ionomycin (data not shown) differed markedly from that for SK&F 96365 (Fig. 1C), in that with ionomycin there was an initial rapid formation of [^{3}H]IP$_3$, consistent with the rapid activation of one or more PLC isozymes. Further, the effects of 30 μM SK&F 96365 and 3 μM ionomycin, concentrations at the top or near the top of the respective concentration-

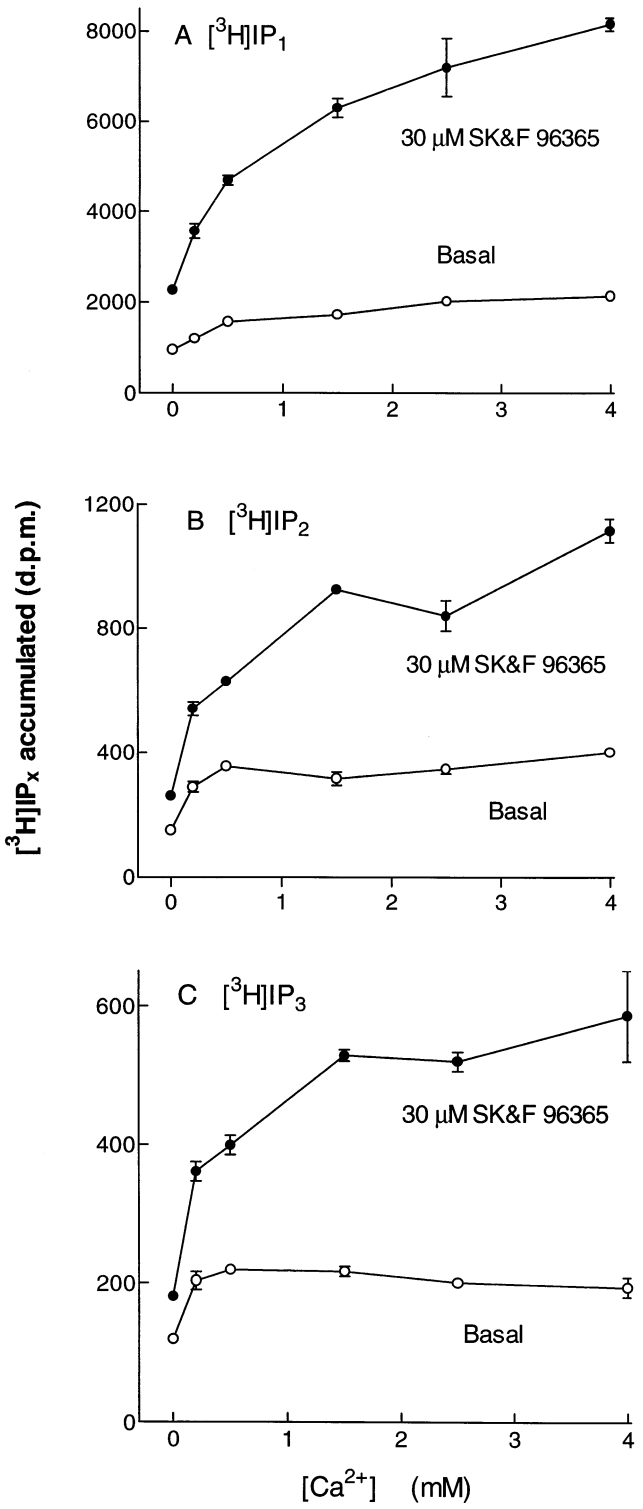

FIG. 3. Ca^{2+}-dependence of SK&F 96365-induced $[^3H]IP_x$ accumulation in U373 MG cells. Points are means ± SEM from triplicate determinations within a single experiment in the presence or absence (basal) of 30 μM SK&F 96365. Where no error bars are shown the error was within the size of the symbol. The whole experiment was repeated twice more. (A) $[^3H]IP_1$ (B) $[^3H]IP_2$ (C) $[^3H]IP_3$. The difference between the stimulated accumulations (SK&F 96365 - basal) at 1.3 and 4 mM Ca^{2+} for $[^3H]IP_1$ in A was statistically significant ($P < 0.05$, Student–Newman–Keuls multiple range test).

response curves, were additive (96 ± 4% of the sum of the responses to each drug acting alone, N = 3).

The possibility remains that SK&F 96365 might induce a local change in $[Ca^{2+}]_i$ immediately below the plasma membrane in close proximity to PLC and Ca^{2+} extrusion processes. In this case there could be stimulation of PLC without any marked change in bulk cytoplasmic $[Ca^{2+}]$. The effect of SK&F 96365 might be mediated by a local depolarisation, an action reported for endothelial cells [8], and activation of a voltage-dependent Ca^{2+} channel. However, increasing extracellular K^+ from 4.5 to 50 mM produced only a small stimulation of $[^3H]IP_1$ accumulation, which was statistically significant only with 40 mM (114 ± 3% of control) and 50 mM K^+ (122 ± 3% of control) (both N = 3; Dunnett test). There is thus no indication of depolarisation-induced Ca^{2+} entry. Stimulated Ca^{2+} entry through *N*-methyl-*D*-aspartate receptor channels is also unlikely, since even in the absence of extracellular Mg^{2+} 100 μM *N*-methyl-*D*-aspartate produced only a small stimulation of $[^3H]IP_1$ accumulation (138 ± 4% of basal, N = 3).

SK&F 96365 caused the release of Ca^{2+} from intracellular stores, as determined by fluorescence measurements, in accord with previous reports [2, 4, 7]. The best-fit EC_{50} for the peak release in U373 MG cells, 26 ± 14 μM, was similar to that for SK&F 96365-stimulated $[^3H]IP_1$ accumulation. However, 5 μM thapsigargin, which produced a larger peak increase in $[Ca^{2+}]_i$ than SK&F 96365, produced only a small stimulation of $[^3H]IP_1$ accumulation in U373 MG cells in normal Ca^{2+}-containing medium, mean 1.7 ± 0.2 fold of basal (N = 3), which was statistically significant in only one experiment. The explanation for the Ca^{2+}-dependence of $[^3H]IP_1$ accumulation induced by SK&F 96365 (Fig. 3) thus remains obscure.

SK&F 96365, a *N*-substituted imidazole, is closely related to the class of antifungal agents typified by miconazole and econazole, which are known to share at least some of the actions of SK&F 96365 on cellular Ca^{2+} handling [2, 5, 17]. This is true for phosphoinositide metabolism in U373 MG cells, since miconazole also stimulated $[^3H]IP_1$ accumulation (maximum response 173 ± 3% of basal, approx. EC_{50} 6.5 ± 1.0 μM; the very hydrophobic character of miconazole makes the actual free concentration uncertain). In view of the apparent selectivity of SK&F 96365 for transformed cells, it is interesting to note that a further *N*-substituted imidazole, lonidamine, has some anti-tumour action [18].

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